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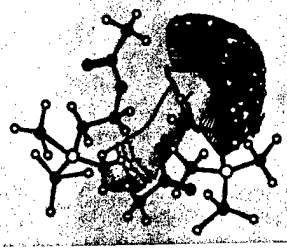

SUBTYPES OF MUSCARINIC RECEPTORS

Proceedings of the Eighth International Symposium on Subtypes of Muscarinic Receptors

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LIFE SCIENCES
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AD_____

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PRINCIPAL INVESTIGATOR: Ruth R. Levine, Ph.D.

CONTRACTING ORGANIZATION: Boston University School of Medicine
Boston, Massachusetts 02118

REPORT DATE: December 1998

TYPE OF REPORT: Final Proceedings

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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EIGHTH SYMPOSIUM ON SUBTYPES OF MUSCARINIC RECEPTORS

FINAL REPORT

RUTH R. LEVINE

January 15, 1999

Supported by

U.S. ARMY MEDICAL RESEARCH ACQUISITION ACTIVITY
Fort Detrick Frederick, Maryland 21701-5012

Grant No. DAMD 17-98-1-8013

Boston University School of Medicine
Boston Massachusetts 02118

Re: DAMD 17-98-1-8013

Final Report

U.S.Army Medical Research Acquisition Activity

Grant No. DAMD 17-98-1-8013

The recently completed Eighth Symposium on Subtypes of Muscarinic Receptors was judged an outstanding success, upholding the standards for excellence set by the seven previous symposia. This judgment was based on: 1) the high quality and timeliness of the invited scientific papers; 2) the large number of first rate and informative posters presented; 3) the invited participation of women and minorities in the scientific sessions; 4) the large number of predoctoral fellows and young scientists whose attendance was made possible by funds granted to the Symposium, and 5) the short oral presentations of selected poster papers.

There were 153 attendees at the Eighth Symposium, half of whom were from 17 countries outside the United States. This attendance figure is most gratifying since, for the first time, the Symposium was not held in conjunction with any other meeting. Twenty-nine (29) major papers were presented in the regular sessions, six of these by women scientists. A total of 78 poster papers were discussed in the two scheduled afternoon sessions, but posters were available for viewing for the entire 4-day meeting. Eight posters were selected for 10 minute oral presentations at an afternoon session set aside for this new feature. It is also noteworthy that the papers selected for oral presentations on the basis of their importance and impact included that of a predoctoral fellow as well as those of three additional female scientists; the choice was made by the Committee in the absence of any prior knowledge of the status of the author.

Among the highlights of the subjects presented in the regular sessions were: the role of muscarinic receptors in genito-urinary smooth muscle function and pathology; the potential role of muscarinic receptors in schizophrenia; preclinical and clinical data on tiotropium in obstructive pulmonary diseases, and the role of muscarinic receptors in nociception and analgesia.

The Proceedings of the Eighth Symposium, which contains the edited manuscripts of the papers presented in the regular sessions as well as the abstracts of the poster papers is to be published in Life Sciences as Volume 64, Numbers 6&7, January 8, 1999 – only 4 months after the close of the Symposium. Proceedings will be sent free of charge to the thousands of subscribers of Life Sciences worldwide as well as to hundreds of others including all of our pre- and postdoctoral fellows. Grant support from the U.S.Army Medical Research Acquisition Activity was largely responsible for the publication of the Proceedings and this has been acknowledged in the program distributed to all attendees and in Life Sciences.

We are extremely pleased that the grants we received and the funds contributed by 17 pharmaceutical companies made it possible for us to pay the travel and accommodation expenses for 28 speakers and committee members from academia. The figure of which we are most proud, however, concerns the large number of pre- and postdoctoral students whom we were able to bring to the Symposium. There were 18 in all and all of these young investigators used this opportunity to present the results of their own research. The attendance of these young scientists was made possible and was largely supported by grants from the National Institute of Neurological Disorders and Stroke and from the National Science Foundation as well as by contributions from industry. A list of the pre- and postdoctoral fellows and their affiliations is appended along with samples of the many letters received indicating the benefits derived from, and the overall success of, the Symposium.

Another measure of success of the Symposium was the high degree of active participation of the registrants in the formal sessions of the Symposium and their interaction in informal gatherings. The attendance at each of the five lecture sessions was excellent and appeared to include all registrants. The 5-10 minute period of discussion between papers was informative and provocative. The excellent lighting and space available for posters encouraged lengthy discussions by both presenters and viewers. There appeared to be much enthusiasm for the research being presented particularly by the young scientists. All in all, it is evident that the Eighth Symposium on Subtypes of Muscarinic Receptors has not only encouraged the research efforts of young investigators but has also stimulated and is stimulating additional research which should, indeed, lead to the development of new and better therapeutic agents as well as agents useful to the U.S. Army Research and Acquisition Activity and to the U.S. Army Medical Research Institute Chemical Defense.

Plans for the Ninth Symposium have been set in motion. The Symposium will be held in the year 2000, this time again in conjunction with the meeting of the Society for Neuroscience. We hope that USAMRAA will again help to support the publication of the Symposium's Proceedings.

Pre- and Postdoctoral Fellows Supported by Symposium

Predoctoral Fellows

1. Jennifer Berkeley
   Dept. of Neurology
   Emory University
   Atlanta, GA

2. Renee Chmelar
   University of Washington
   Seattle, WA

3. Adolfo Cuadra
   Neuroscience Research in Psychiatry
   University of Minnesota
   Minneapolis, MN

4. Gurpreet Kaur
   Faculty of Pharmacy
   University of Toronto
   Toronto, Canada

5. Katharine Lee
   Dept. of Molecular Pharmacology & Biological Chemistry
   Northwestern University
   Chicago, IL

6. Kedan Lin
   Dept. of Biopharmaceutical Sciences and Pharmaceutical Chemistry
   UCSF
   San Francisco, CA

7. Diane Porter
   Dept. of Pharmacology
   Penn State University
   Hershey, PA

8. Mac Priebe
   Dept. of Psychology
   University of Toronto
   Toronto, Canada

9. Gregory Sawyer
   Dept. of Pharmacology
   UC, Irvine
   Irvine, CA

10. Scott Sorensen
    Neuroscience Lab.
    University of Michigan
    Ann Arbor, MI

11. Chi-Shing Sum
    Faculty of Pharmacy
    University of Toronto
    Toronto, Canada

12. Stuart Ward
    National Institute for Medical Research
    London, U.K.

13. Yi Zhang
    Dept. of Pharmacology
    East Tennessee State University
    Johnson City, TN

Postdoctoral Fellow

1. Alan Braverman (also an invited speaker)
   Temple University
   Philadelphia, PA

2. Arthur Christopoulos
   Neuroscience Research in Psychiatry
   University of Minnesota
   Minneapolis, MN

4. Abulkhair Mamoon
   Dept. of Pharmacology & Toxicology
   University of Mississippi
   Jackson, MS

5. Susan Rouse
   Dept. of Pharmacology
   Emory University
   Atlanta, GA

REPORT OF INVENTIONS AND SUBCONTRACTS

(Pursuant to "Patent Rights" Contract Clause) (See Instructions on Reverse Side.)

Form Approved
OMB No. 0704-0297
Expires Jun 30, 1992

1a. NAME OF CONTRACTOR / SUBCONTRACTOR	c. CONTRACT NUMBER	2a. NAME OF GOVERNMENT PRIME CONTRACTOR	c. CONTRACT NUMBER	3. TYPE OF REPORT *(X one)*
				a. INTERIM ☐ b. FINAL ☐
b. ADDRESS *(Include ZIP Code)*	d. AWARD DATE *(YYMMDD)*	b. ADDRESS *(Include ZIP Code)*	d. AWARD DATE *(YYMMDD)*	4. REPORTING PERIOD *(YYMMDD)*
				a. FROM
				b. TO

SECTION I – SUBJECT INVENTIONS

5. "SUBJECT INVENTIONS" REQUIRED TO BE REPORTED BY CONTRACTOR / SUBCONTRACTOR *(If "None," so state)*

a. NAME(S) OF INVENTOR(S) *(Last, First, MI)*	b. TITLE OF INVENTION(S)	c. DISCLOSURE NO., PATENT APPLICATION SERIAL NO. OR PATENT NO.	d. ELECTION TO FILE PATENT APPLICATIONS				e. CONFIRMATORY INSTRUMENT OR ASSIGNMENT FORWARDED TO CONTRACTING OFFICER	
			(1) United States		(2) Foreign			
			(a) Yes	(b) No	(a) Yes	(b) No	(1) Yes	(2) No
	NONE							

f. EMPLOYER OF INVENTOR(S) NOT EMPLOYED BY CONTRACTOR / SUBCONTRACTOR		g. ELECTED FOREIGN COUNTRIES IN WHICH A PATENT APPLICATION WILL BE FILED	
(1) (a) Name of Inventor *(Last, First, MI)*	(2) (a) Name of Inventor *(Last, First, MI)*	(1) Title of Invention	(2) Foreign Countries of Patent Application
(b) Name of Employer	(b) Name of Employer		
(c) Address of Employer *(Include ZIP Code)*	(c) Address of Employer *(Include ZIP Code)*		

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a. NAME OF SUBCONTRACTOR(S)	b. ADDRESS *(Include ZIP Code)*	c. SUBCONTRACT NO.(S)	d. DFAR "PATENT RIGHTS"		e. DESCRIPTION OF WORK TO BE PERFORMED UNDER SUBCONTRACT(S)	f. SUBCONTRACT DATES *(YYMMDD)*	
			(1) Clause Number	(2) Date *(YYMM)*		(1) Award	(2) Estimated Completion
NONE							

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a. NAME OF AUTHORIZED CONTRACTOR / SUBCONTRACTOR OFFICIAL *(Last, First, MI)*	c. I certify that the reporting party has procedures for prompt identification and timely disclosure of "Subject Inventions," that such procedures have been followed and that all "Subject Inventions" have been reported.	
b. TITLE	d. SIGNATURE	e. DATE SIGNED

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH ACQUISITION ACTIVITY
820 CHANDLER STREET
FORT DETRICK, MARYLAND 21702-5014

REPLY TO
ATTENTION OF:

December 9, 1998

Special Projects Branch/WAC/ljm

SUBJECT: Grant Number DAMD17-98-1-8013

Dr. Ruth R. Levine
Boston University School
of Medicine
Boston University Medical Center
80 East Concord Street
Boston, Massachusetts 02118

Dear Dr. Levine:

Reference is made to Grant Number DAMD17-98-1-8013. This grant expired on 30 October 1998 and our records show that the items checked below are required for grant completion.

a. (X) Final Report, was due 30 November 1998. In accordance with Article 8 of this grant, forward one (1) original and two (2) copies of the required final report.

b. (X) Final inventory of all property acquired under the grant. Recommendations for disposition may accompany the inventory. This may be submitted on your letterhead.

c. (X) Final Patent Report (2 copies, form enclosed). Complete the Final Patent Report even if it is negative. If you apply for a patent, forward a copy of the patent application and the confirmatory license to this office. In filing your patent, ensure that the following statement appears on the application:

"The U.S. Government has a nonexclusive, non-transferable, irrevocable paid-up license to practice or have practiced this invention for or on its behalf as provided for by the terms of Grant Number DAMD17-98-1-8013 awarded by the U.S. Department of the Army."

d. (X) SF 272 Federal Cash Transaction Report. Any unexpended funds shall be returned to the Government. Submit a check made out to the U.S. Treasury with the SF 272.

In addition, if you have been audited in accordance with OMB Circular A-110, please submit copies of audits covering the term of the grant.

The enclosed forms must be completed and returned in order to closeout the grant file. Therefore, you are requested to return the forms by 11 January 1999. Please submit all documentation to:

U.S. Army Medical Research Acquisition Activity
ATTN: MCMR-AAA-V (Closeout)
820 Chandler Street
Fort Detrick, MD 21702-5014

If you have further questions or concerns about the closeout documentation, please contact Rosalie A. Putman, Contract Specialist, at (301) 619-7348, FAX (301) 619-2505, E-mail: rosalie.putman@det.amedd.army.mil.

Sincerely,

Linda J. Mandeville
Procurement Technician

Enclosures

cf: MCMR-AAA-V (Closeout)

UNIVERSITY OF CAMBRIDGE
DEPARTMENT OF PHARMACOLOGY
Tennis Court Road, Cambridge CB2 1QJ

Telephone (0223) 334000
Fax (0223) 334040

Dr. Ruth R Levine,
Boston University School of Medicine 8/9/98

My dear Ruth,

I have just returned after our visit to the Symposium and our subsequent trip to the Cape with Olga's sister Judith; we were blessed with splendid weather and stayed at a lovely place.

I thought that the Muscarinic symposium was very successful and there were some exciting new developments; there is clearly plenty of life in it to make a continuation worthwhile. The organisation was, as usual, perfect as we expect from any thing Ruth organises.

It must have been a terrible burden for you to keep your mind on this with Martin being in such a poor state. I do hope that things are improved and indeed that there is some prospect of his return home before too long; please do give him the very best wishes from Olga and me.

With all the very best to you,

As ever,

Arnold

MRC COLLABORATIVE CENTRE

1-3 Burtonhole Lane, Mill Hill, London NW7 1AD
Tel: (+44) 0181 906 3811 Fax: (+44) 0181 906 1395

Collaborative Centre
MRC

Ruth Levine, Ph.D.
Associate Dean, Emerita
Boston University School of Medicine
715 Albany Street
Boston
MA 02118-2526
USA

4th Sept. 1998

Dear Ruth,

This is a small 'thank you' for your efforts and courage in the organisation and execution of the Muscarinic Subtypes meeting. It may be my last Muscarinic Subtypes meeting (my contract finishes shortly) but I hope it won't be yours. It was a wonderful conference for me, both scientifically and socially. It was good to meet old friends, and colleagues known from the literature, and the extended poster sessions allowed more time to meet with the youngsters, which I enjoyed. And these youngsters love you. At the end of the meeting you may know that you received a standing ovation; what you may not know (I was watching, and your eyes were averted) is that everyone was smiling - it was a joyous acclamation.

I would like to make a couple of suggestions.

1) I think that it would be enjoyable and useful to include a touristic period in the conference - this could be a bus tour of the host city, occupying the last afternoon of the meeting so that people could easily avoid it.

2) Even with two afternoons devoted to posters, it was not possible to view all the ones of interest while the authors were present. In order to facilitate communication between attendees I suggest a local network utilising Internet protocols, with each attendee having their own address and home page. Ideally there should be a terminal in each room allowing access to the network, access to a local floppy disk, and access to a local (or central) printer. A few terminals should be in the poster area.

Many thanks,

Sel

Sebastian Lazareno

Richard M. Eglen, Ph.D.
Vice President & Director
Center For Biological Research
Neurobiology Unit
Phone: 650-354-7239
Fax: 650-852-3111
E-mail: richard.eglen@roche.com

Roche

Bioscience

September 8, 1998

Ruth R. Levin, Ph.D.
Professor Emerita of Pharmacology
715 Albany Street
Boston, MA 02118-2526

Dear Ruth,

Just a brief note on behalf of my group and I, to express our thanks for organizing such a stimulating and informative meeting at the Ferncroft. It was very enjoyable and I look forward to the next one in two years. I would also like to thank you for the invitation to join the scientific organizing committee and look forward to speaking with you about the next steps in the future.

I hope all is well with you and your family.

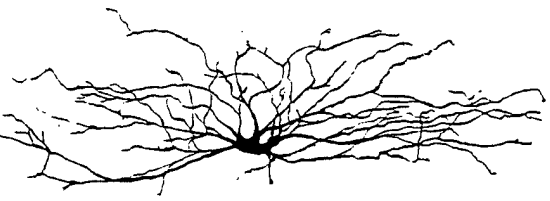
Best regards,

Richard

Richard M. Eglen

Phone: (313) 763-4268
Fax: (313) 936-2690

The University of Michigan
Neuroscience Laboratory Building



September 3, 1998

Dr. Ruth Levine
Professor Emerita of Pharmacology
Associate Dean, Emerita
Boston University School of Medicine
715 Albany Street
Boston, MA 02118-2526

Dear Ruth:

I wanted to express my sincere thanks to you for once again staging an outstanding and successful Symposium. Those of us who have arranged such events in the past are very cognizant of the amount of time and commitment that such a venture takes and are thus doubly appreciative of your efforts. I know that I speak for all of the attendees when I say that the whole event was most enjoyable and very beneficial intellectually.

I also would like to thank you for your continued support of my graduate students and in facilitating their attendance at the meeting. As you are aware, I have had a long-standing interest in muscarinic receptors and their coupling to phosphoinositide turnover and my R01 grant on this topic is about to begin its 12th year of funding. I have been most fortunate to have had some outstanding students choose my laboratory to pursue their Ph.D. degrees You may remember Diana Slowiejko (who attended the Newport Beach and Ft. Lauderdale meetings), Daniel Linesman (who attended the Vienna meeting) and now Scott Sorensen, who has, through your generosity, been able to attend the last two Subtypes meetings. It's undoubtedly true that without your support, the level of their participation in the Meetings would have been considerably reduced. All of us owe you a debt of gratitude for your continued interest in graduate student education.

Both Anne and I realize that the Meeting occurred at a time that was most difficult for your personally. We sincerely hope that your husband's health improves in the near future. We also look forward to the Ninth International Meeting! Once again, many thanks for our hard work. All best wishes,

Sincerely yours,

Steve

Stephen K. Fisher, Ph.D.
Professor of Pharmacology

SKF:jk

1103 East Huron Street Ann Arbor, Michigan 48104-1687

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2 September 1998

Dr. Ruth R. Levine
Professor Emerita of Pharmacology
Boston University School of Medicine
715 Albany Street
Boston, MA 02118-2526

Dear Ruth,

Congratulations on your superb organization of the Eighth International Symposium on Subtypes of Muscarinic Receptors! The meeting succeeded in large measure because of your seemingly tireless efforts to gather a group of lively scientists to discuss the most recent advances in this field. As such, this meeting adds another pearl to your string of successes in establishing and sustaining this symposium as a forum for scientific exchange.

The gathering had the flavor and spirit of a Gordon Conference as most of the participants were able to attend the entire meeting and be available for intimate discussions and provocative speculation. The lodging accommodations and the meal arrangements were conducive to the spirit of inquiry that permeated the symposium. I especially enjoyed the oral presentations by Drs. A. Fryer, W.C. Degroat and D. Saffen. This is in no way a slight at the other speakers who did very well. The afternoon session of selected poster presentations was also well done.

I have one negative remark and that concerns the presentation by Dr. R. Kelly who did little more than appear for his oral paper presentation. He foreclosed extensive discussion by announcing that his appearance was an interruption of another meeting to which he had to return immediately. He avoided answering questions and did not stay even through lunch so as to preclude more personal discussion. This conduct is contrary to the spirit of the meeting and must be discouraged.

On balance, the positive aspects of the symposium carried the week and we are all indebted to you for your gracious and generous support of scholarship in the service of science. I look forward to the excitement of the next gathering.

Good wishes.

Sincerely,

Achilles

Achilles Pappano
Professor

Phone: (313) 763-4268
Fax: (313) 936-2690

The University of Michigan
Neuroscience Laboratory Building

September 3,1998

Dr. Ruth R. Levine
Professor Emerita of Pharmacology
Boston University School of Medicine
Boston University Medical Center
80 East Concord Street
Boston, MA 02118

Dr. Levine,

Thank you for your efforts as the coordinator of the Eighth Symposium on Subtypes of Muscarinic Receptors. I recently coordinated a small interdepartmental symposium at the University of Michigan and can only begin to imagine the amount of effort expended to run an international meeting. You have done a magnificent job once again. As a student, I especially enjoyed the one-on-one interactions with the scientists in attendance and I was able to discuss several post-doctoral opportunities as well. I would also like to thank you for your generous financial support which provided for a rewarding learning experience. I look forward to seeing you at the next symposium.

Sincerely,

Scott Sorensen

Scott Sorensen

1103 East Huron Street

Ann Arbor, Michigan 48104-1687

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

National Institutes of Health
Bethesda, Maryland 20892

Aug. 31, 1998

Dr. Jürgen Wess
Laboratory of Bioorganic Chemistry
NIDDK, National Institutes of Health
Bldg. 8A, Room B1A-09
Bethesda, MD 20892

Tel. 301-402-3589
Fax 301-402-4182

Dr. Ruth R. Levine
Symposium Coordinator
Boston University School of Medicine
715 Albany Street
Boston, MA 02118

Subj.: Eight International Symposium on Subtypes of Muscarinic Receptors
(Danvers, MA, Aug. 98)

Dear Ruth,

Thanks again for the wonderful job you did in organizing this year's "Muscarinic Receptor Meeting". My coworkers and I very much enjoyed both the high scientific quality of this meeting as well as the various social events.

Enclosed please find the receipt for my flight ticket ($ 84.00)(Washington, D.C./Dulles - Boston; round trip).

Thanks again for everything. I am already looking forward to the next meeting in two years from now.

Best wishes!

Yours sincerely,

Jürgen

Johann Wolfgang Goethe-Universität
Frankfurt/Main

Prof. Dr. Günter Lambrecht

Department of Pharmacology
Biocentre Niederursel
University of Frankfurt

Marie-Curie-Straße 9
Gebäude N260
D-60439 Frankfurt
Germany

Tel.: #(0)69-798-29366
Fax.: #(0)69-798-29374
Lambrecht@em.uni-frankfurt.de

September 4, 1998

Dr.
Ruth R. Levine
Boston University
School of Medicine
715 Albany Street, L310
Boston, MA 02118
U S A

Dear Ruth,

This is to offer my sincere thanks for having me at the Eighth Symposium on Subtypes of Muscarinic Receptors in Danvers. I also want to take this opportunity to extend my sincerest thanks to you for organizing this meeting. I think it was again a highly successful conference, and I enjoyed the meeting.

Thank you again for all your efforts.

With best wishes, also from Heidi,
Cordially,

Günter Lambrecht

P.S.:
Please find enclosed my signed Travel Expense Report, together with a copy of my plane ticket and receipts for transfer Logan - Danvers - Logan.

UNIVERSITY OF WASHINGTON SCHOOL OF MEDICINE

Renée Chmelar
University of Washington
Box 357750
Department of Pharmacology
Seattle, WA 98195

Ruth R. Levine, Ph.D.
Boston University
School of Medicine
80 East Concord Street
Boston, MA 02118

Dear Dr. Levine:
I wanted to express to you my appreciation for making my participation in the Eighth International Symposium on the Subtypes of Muscarinic Receptors financially possible. As a graduate student I deeply appreciate the opportunity to attend this meeting which furthers my knowledge of the field in which I work and allows me to meet the scientists with whose work I have become familiar. The meeting was both intellectually rewarding and motivational!

Thank you again for making my attendance at the meeting possible.

Sincerely,

Renée Chmelar

September 21, 1998

Dr. Levine,

I am writing to express my thanks to you for having me as a Fellow at the Eighth Symposium on Subtypes of Muscarinic Receptors. It was obvious that you put in a lot of hard work and it was greatly appreciated. I am also glad I was able to help with the audio-visual jobs during the Oral Presentations.

I would also like to thank you for providing me with an alternative dinner at the Lobster Dinner. Having a food allergy is hard and often frustrating. However, you did your best in making sure I was accommodated. I truly appreciate that!

Again, your hard work was greatly appreciated, for the meeting was a huge success. As a third year graduate student, it was a very motivating and enjoyable experience.

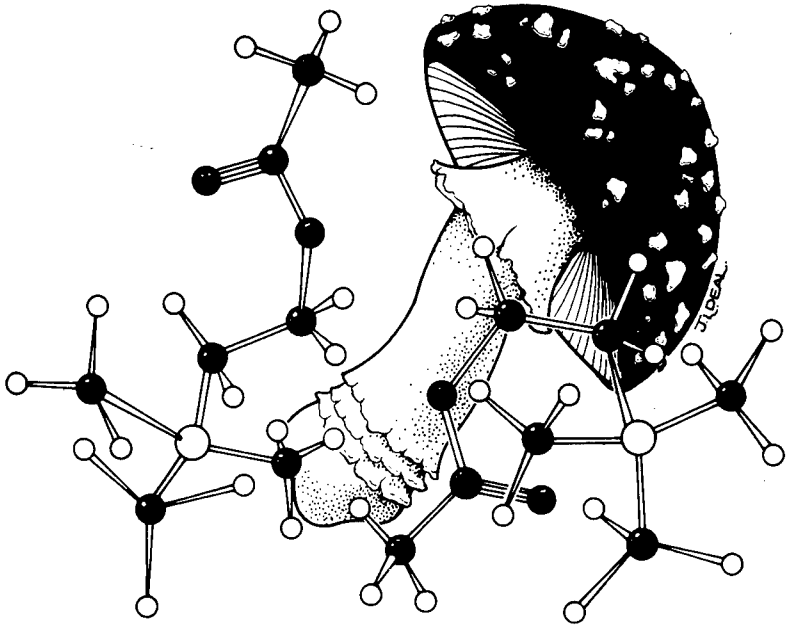
Thank you,

Diane Porter

Diane Porter
Graduate Student, Penn State University

SUBTYPES OF MUSCARINIC RECEPTORS

Proceedings of the Eighth International Symposium on Subtypes of Muscarinic Receptors



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AUGUST 25—29, 1998
DANVERS, MASSACHUSETTS, USA

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STRUCTURE-FUNCTION ANALYSIS OF MUSCARINIC RECEPTORS AND THEIR ASSOCIATED G PROTEINS

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Summary

Each member of the muscarinic receptor family (M1-M5) can interact only with a limited subset of the many structurally closely related heterotrimeric G proteins expressed within a cell. To understand how this selectivity is achieved at a molecular level, we have used the $G_{i/o}$-coupled M2 and the $G_{q/11}$-coupled M3 muscarinic receptors as model systems. We developed a genetic strategy involving the coexpression of wild type or mutant muscarinic receptors with hybrid or mutant G protein α subunits to identify specific, functionally relevant receptor/G protein contact sites. This approach led to the identification of N- and C-terminal amino acids on α_q and α_i that are critical for maintaining proper receptor/G protein coupling. Moreover, several receptor sites were identified that are likely to be contacted by these functionally critical Gα residues. To gain deeper insight into muscarinic receptor structure, we recently developed a cysteine disulfide cross-linking strategy, using the M3 muscarinic receptor as a model system. Among other structural modifications, this approach involves the removal of most native cysteine residues by site-directed mutagenesis, the insertion of three factor Xa cleavage sites into the third intracellular loop, and systematic 'reintroduction' of pairs of cysteine residues. Following treatment of receptor-containing membrane preparations with factor Xa and oxidizing agents, disulfide cross-linked products can be identified by immunoprecipitation and immunoblotting studies. This approach should greatly advance our knowledge of the molecular architecture of muscarinic and other G protein-coupled receptors.

Key Words: mutagenesis studies, G protein-coupled receptors, G protein α-subunits, receptor/G protein coupling, disulfide cross-linking studies

The five muscarinic acetylcholine receptors (M1-M5) are prototypical members of the superfamily of G protein-coupled receptors (GPCRs) (1-3). Like most other GPCRs, each muscarinic receptor subtype shows a high degree of G protein coupling selectivity. The M1, M3, and M5 receptors preferentially couple to G proteins of the $G_{q/11}$ class, whereas the M2 and M4 receptors are selectively linked to G proteins of the $G_{i/o}$ family (1-3). To understand how this selectivity is achieved at a molecular level is a major focus of ongoing research efforts in our laboratory. Systematic mutagenesis studies have led to the identification of a series of residues (located within the second (i2) and third (i3) intracellular loops) that play key roles in determining the G protein coupling profiles of individual muscarinic receptor subtypes (reviewed in ref. 3). Despite this knowledge, however, the molecular architecture of the receptor/G protein complex still remains only poorly defined. Clearly, the delineation of accurate molecular models of the receptor/G protein interface requires the identification of sites on the G

protein(s) that are contacted by functionally critical receptor residues.

Residues involved in M2 receptor/$G_{i/o}$ coupling selectivity

A considerable body of evidence indicates that the C-terminal five amino acids of Gα subunits play a key role in receptor recognition (4-9). We have recently shown, for example, that the Gi/o-coupled M2 muscarinic receptor (which does not efficiently interact with wild type (wt) α_q) can productively couple to mutant α_q subunits in which the last five amino acids of α_q are replaced with the corresponding α_i or α_o sequences (5, 8). Coexpression of such mutant G protein subunits with a large number of M2/M3 hybrid muscarinic receptors showed that the ability of the M2 receptor to recognize the C-terminus of $\alpha_{i/o}$ subunits is dependent on the presence of a group of residues (Val385, Thr386, Ile389, and Leu390; "VTIL motif") located at the junction between the i3 loop and transmembrane domain VI (TM VI) (refs. 5, 8).

We subsequently employed a gain-of-function mutagenesis strategy to identify individual amino acids within the C-terminal tail of $\alpha_{i/o}$ subunits that are critical for recognition by the M2 muscarinic receptor (8). Coexpression of a series of C-terminally modified mutant α_q subunits with the M2 receptor led to the identification of two mutant α_q subunits containing single α_q-> α_i point mutations at their C-terminus which, in contrast to wt α_q, were able to productively interact with the M2 receptor. This study provided the first example where the receptor coupling selectivity of G protein α subunits could be altered by single amino acid substitutions (8). The α_i residues present in the two mutant α_q constructs capable of efficiently interacting with the M2 muscarinic receptor were the -4 cysteine and the -3 glycine, respectively. These two residues are well conserved only within the $\alpha_{i/o}$ family. Moreover, functional analysis of the receptor coupling properties of additional α_q/α_i mutant subunits suggested that the C-terminal aromatic residue (phenylalanine or tyrosine) characteristic for $\alpha_{i/o}$ subunits also contributes to the selectivity of receptor recognition (8).

Residues involved in M3 receptor/$G_{q/11}$ coupling selectivity

To examine whether the results obtained with the $G_{i/o}$-coupled M2 muscarinic receptor are more generally applicable, we extended the coexpression strategy described in the previous section to other functional classes of GPCRs and Gα subunits. We initially demonstrated that the rat M3 muscarinic receptor, while unable to efficiently interact with wt α_s, can productively couple to a mutant version of α_s (sq5) in which the last five amino acids were replaced with the corresponding α_q sequence (9). This observation is consistent with the concept that the C-terminus of Gα subunits is generally important for the selectivity of receptor recognition.

To identify the region on the M3 receptor that can interact with the C-terminus of α_q, we again employed a coexpression strategy involving the use of M2/M3 hybrid muscarinic receptors (note that neither of the two wt receptors can efficiently couple to wt α_s) and the mutant α_s subunit, sq5 (ref. 9). A mutant M2 receptor in which the functionally critical VTIL motif at the i3 loop/TM VI junction was replaced with the corresponding M3 receptor sequence (Ala488, Ala489, Leu492, and Ser493; "AALS motif") was unable to productively interact with wt α_s but gained the ability to couple to sq5. This observation indicated that the C-terminus of the i3 loop is generally important for recognition of the C-terminus of Gα subunits. Surprisingly, however, two additional mutant M2 receptors, which contained M3 receptor sequences in the i2 loop and at the N-terminus of the i3 domain, respectively, were identified that also gained coupling to sq5 (ref. 9). The i2 loop as well as the N- and C-terminal segments of the i3 domain are known to contain key residues determining the $G_{q/11}$ coupling selectivity of the M3 muscarinic receptor (3). These residues are predicted to cluster together in a "triangle" defined by the positions of TM III, V, and VI, thus forming a well-defined G protein binding site (10, 11). One possible explanation for the observed ability of multiple M2/M3 hybrid

receptors to interact with the sq5 subunit therefore is that the C-terminal portion of α_q can simultaneously contact multiple receptor residues forming this G protein binding surface.

We next investigated which specific amino acids located within the C-terminal segment of α_q are of primary importance for the selectivity of M3 receptor/G_q interactions (9). Toward this goal, single amino acids at the C-terminus of α_s were replaced with the corresponding α_q residues, and the resulting mutant G proteins were studied for their ability to gain coupling to the M3 muscarinic receptor (using cotransfected COS-7 cells). This analysis led to the identification of two α_s->α_q single point mutants which gained the ability to functionally interact with the M3 muscarinic as well as with other $G_q/11$-coupled receptors (9). The α_q residues present in these constructs were a -5 glutamate and a -3 asparagine, respectively, which are found in all members of the $\alpha_q/11$ protein family. It is likely that these two α_q residues are generally important for selective recognition by $G_q/11$-coupled receptors, probably by directly contacting the receptor proteins. The side chain of the -3 asparagine may be able to hydrogen-bond back to the main chain, thus forming a type VIII β-turn characterized by a spatial arrangement of the C-terminal $\alpha_q/11$ residues which differs from that found with $\alpha_{i/o}$ subunits (12).

These findings also indicate that the precise positions of the C-terminal Gα residues that are involved in determining the selectivity of receptor/G protein interactions vary between different functional classes of Gα subunits. However, in both $\alpha_{i/o}$ and $\alpha_q/11$ subunits, the -3 residue is of fundamental importance for proper receptor recognition (8, 9). As a general rule, the residues present at this position are perfectly conserved within individual Gα subfamilies and can correctly predict the coupling profile of a given Gα subunit.

Importance of the N-terminus of $G\alpha_q$ for receptor coupling selectivity

Although it is clear that the C-terminal regions of Gα are critically involved in receptor/G protein coupling (see previous section), other regions of Gα may also contribute to receptor binding and the selectivity of receptor/G protein interactions. Specifically, several lines of evidence suggest that the N-terminal portion of Gα may be in contact with the receptor protein (13-15).

To test the hypothesis that the extreme N-terminus of $\alpha_{i/o}$ subunits also plays a role in determining the selectivity of receptor/G protein interactions, we initially created a hybrid Gα subunit in which the first ten amino acids of α_q, including a six-amino-acid extension that is characteristic for $\alpha_q/11$ subunits, were replaced with the N-terminal four amino acids (MGCT) present in α_i/α_o subunits (referred to as i4q; Fig. 1A). As a control, we also generated a mutant α_q subunit, referred to as -6q, that lacked the six-amino-acid extension present in α_q and α_{11} (Fig. 1A). We then examined, in cotransfected COS-7 cells, whether the $G_{i/o}$-coupled M2 muscarinic receptor would gain the ability to interact with these mutant G proteins (16). To our surprise, these studies showed that the M2 receptor gained the ability to couple to both i4q as well as -6q, as measured by carbachol-dependent stimulation of phosphoinositide hydrolysis. This observation suggested that the ability of the M2 receptor to interact with the hybrid i4q subunit is primarily due to the lack of the six-amino-acid extension rather than the presence of four amino acids of α_i sequence. We subsequently showed that several other $G_{i/o}$- as well as G_s-coupled receptors were also capable of interacting with the -6q subunit (but not with wt α_q) (16). These findings suggested that the N-terminal extension characteristic for $\alpha_q/11$ subunits plays an important role in restraining the receptor coupling selectivity of these G proteins.

To study which specific amino acids within the N-terminal segment of $\alpha_q/11$ are critical for constraining receptor coupling selectivity, we next subjected this region to systematic deletion and alanine scanning mutagenesis (17). We prepared two series of N-terminally modified mutant α_q subunits: In one series, the N-terminus of α_q was progressively shortened by deletion mutagenesis, and in the other, amino acids 2-7 were replaced, either individually or in combination, with alanine residues (Fig. 1A). All wt and mutant α_q subunits contained an internal hemagglutinin (HA) epitope

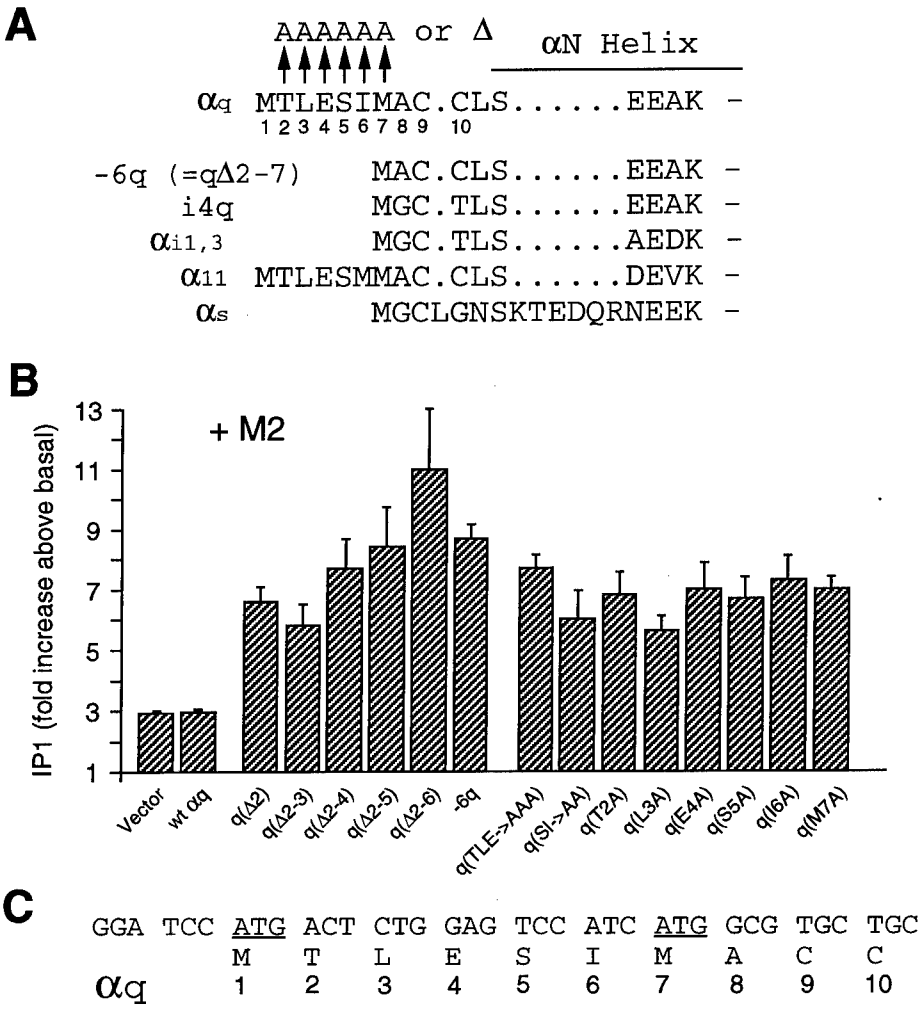

Fig. 1

Mutational analysis of the N-terminal segment of α_q. (A) Amino acids located at the N-terminus of α_q (mouse) (marked with arrows) were deleted or replaced, either individually or in combination, with alanine residues. In i4q, the first ten amino acids of α_q were replaced with the first four amino acids of α_i. For comparison, the N-terminal portions of α_{11} and α_s are also shown. Gaps were introduced to allow for maximum sequence identity. The position of the N-terminal segment of the αN helix, as revealed by X-ray crystallography (29, 30), is indicated. (B) Functional coupling of the M2 muscarinic receptor to mutant α_q subunits. COS-7 cells were cotransfected with expression plasmids coding for the human M2 receptor and vector DNA (pcDNAI), wt α_q, or different α_q mutant subunits. Increases in intracellular IP_1 levels (in the presence of the muscarinic agonist, carbachol (0.5 mM), were determined as described (17) (means ± S.E.M; n=3). (C) Nucleotide sequence of the N-terminal segment of the wt α_q construct used in ref. 17. The two N-terminal in-frame ATG codons (codons one and seven) are underlined.

tag which did not affect the receptor and effector coupling properties of wt α_q (4, 5, 18). Western blot analysis showed that all mutant α_q subunits were properly expressed, at levels similar to or somewhat lower than observed with wt α_q (17). Surprisingly, coexpression studies showed that the M2 muscarinic receptor gained the ability to productively couple to all 14 mutant α_q subunits, resulting in a 6-11-fold stimulation of inositol phosphate production (Fig. 1B). Essentially similar findings were obtained with the $G_{i/o}$-linked D2 dopamine and the G_s-coupled β2-adrenergic receptors (17).

Analysis of the nucleotide sequence coding for the N-terminal segment of α_q shows that the initiating ATG codon (codon one) is followed by a second in-frame methionine codon (codon seven) (Fig. 1C). We also noted that the second ATG codon is present in a better context for translation initiation than the first one (19, 20). We therefore needed to exclude the possibility that codon seven is used as an alternative translation start site. If this were the case, then all mutant α_q constructs would direct the synthesis of the functionally promiscuous -6q subunit (Fig. 1B). To address this issue, we introduced frameshift mutations (single base insertions or deletions) between codons one and seven into two functionally promiscuous mutant α_q constructs (q(L3A) and q(E4A); Fig. 1A). If codon seven is in fact used as a translation start site, these frameshift mutations are predicted not to interfere with the synthesis of -6q but should prevent the synthesis of a functional protein if translation starts from codon one. Coexpression studies and immunoblotting experiments clearly demonstrated that methionine codon seven is not used for translation initiation, at least not to a detectable degree (17). Translation initiation from an alternative (internal) start site can therefore be excluded as a potential cause for the observed functional promiscuity of the studied mutant α_q subunits.

Like most other Gα subunits, α_q and α_{11} are known to be palmitoylated at N-terminal cysteine residues (corresponding to Cys9 and Cys10 in Fig. 1; refs. 18, 21-25). We therefore wanted to examine whether the promiscuous mutant α_q subunits differed from wt α_q in their palmitoylation patterns. Toward this goal, wt α_q and selected mutant α_q subunits were expressed in COS-7 cells, metabolically labeled with [^{3}H]palmitic acid, followed by immunoprecipitation, SDS-PAGE, and fluorography (17). These studies showed that wt α_q and all mutant α_q subunits incorporated significant amounts of [^{3}H]palmitate. However, we also noted that the strength of the palmitoylation signal was generally weaker (by about 40-75%) in the case of the mutant α_q subunits (as compared with wt α_q) (17).

Taken together, these data are consistent with the notion that the six-amino acid extension characteristic for $\alpha_{q/11}$ subunits forms a tightly folded protein subdomain that is critical for regulating the receptor coupling selectivity of these subunits. One possibility is that this subdomain has a gate function by selectively preventing access of $G_{i/o}$- and G_s-coupled receptors. It is also conceivable that the six-amino acid extension exerts indirect conformational effects on other regions of $\alpha_{q/11}$, (e.g. on C-terminal segments) that are crucial for maintaining the receptor selectivity of these subunits. Moreover, our data raise the possibility that receptor coupling selectivity may be regulated by the palmitoylation pattern of Gα subunits.

Development of a strategy to study GPCR structure via disulfide cross-linking

Currently, high-resolution structural information is not available for any GPCR. We therefore decided to devise a cysteine disulfide cross-linking strategy to gain deeper insight into GPCR structure, using the rat M3 muscarinic receptor as a model system. To be able to reliably detect the M3 receptor protein on Western blots, the following structural modifications were made: a) an HA epitope tag was added to the N-terminus of the receptor, b) all five potential N-glycosylation sites present in the N-terminal portion of the receptor protein were eliminated by site-directed mutagenesis, and c) most of the i3 loop, except for the N- and C-terminal 22 amino acids, was deleted (Fig. 2). The resulting mutant receptor (referred to as M3') showed ligand binding and G protein coupling properties similar to the wt M3 receptor (F.-Y. Zeng and J. Wess, unpublished results). However, in contrast to the wt receptor, the M3' receptor could be readily detected via immunoblotting using either a monoclonal antibody directed

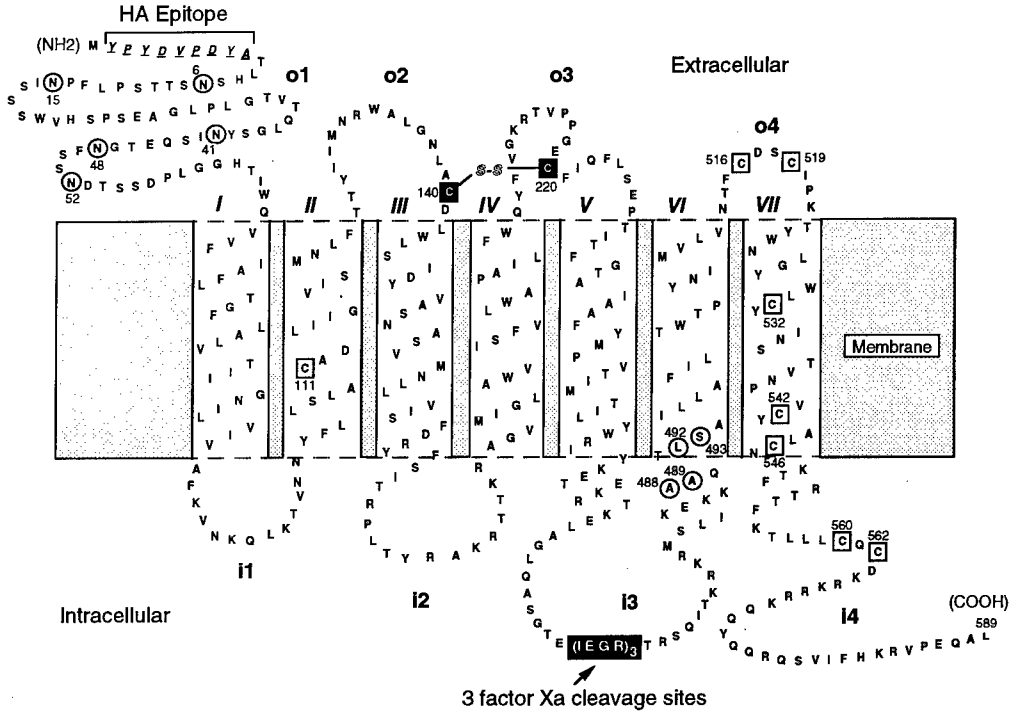

Fig. 2

Transmembrane topology of the rat M3 muscarinic receptor. To be able to detect the M3 receptor protein via different immunological techniques, an HA epitope tag (underlined) was added to the N-terminus, and the five potential N-glycosylation sites present within the o1 region (Asn6, Asn15, Asn41, Asn48, and Asn52) were replaced with glutamine residues. Cysteine residues are shown boxed. Cys140 and Cys220 (highlighted in black) are predicted to be linked via a disulfide bridge (26-28). To be able to study the structure of the M3 muscarinic receptor by using a cysteine disulfide cross-linking strategy (see text for details), the central portion of the i3 loop (196 amino acids) was replaced with three factor Xa cleavage sites. Four functionally critical amino acids located at the i3 loop/TM VI junction are also highlighted (circles; see text for details). Numbers refer to amino acid positions in the rat M3 muscarinic receptor sequence (31).

against the N-terminal HA tag or a polyclonal antibody raised against a peptide corresponding to the last 18 amino acids of the M3 receptor protein. We therefore chose the M3' receptor as a template for further site-directed mutagenesis studies.

We next created three mutant M3 receptors (by replacing native cysteine residues in the M3' receptor with either alanine or serine) which contained no, two (Cys140 and Cys220), or three (Cys140, Cys220, and Cys532) remaining cysteine residues (resulting in M3'(C-less), M3'(C2), and M3'(C3), respectively). It should be noted that Cys140 and Cys220 are predicted to be linked via a disulfide bond (26-28) which appears to be required for proper trafficking of the M3 receptor protein to the cell surface (F.-Y. Zeng, A. Soldner, T. Schöneberg and J. Wess; unpublished results). The completely Cys-free receptor (M3'(C-less)) did not show any radioligand binding or functional activity. However, both the M3'(C2) and M3'(C3) mutant receptors still retained the ability to stimulate carbachol-

dependent inositol phosphate production to the same maximum extent as the wt M3 receptor (F.-Y. Zeng, A. Soldner, and J. Wess; unpublished results). Moreover, the M3'(C3) mutant receptor was able to bind muscarinic agonists and antagonists with affinities similar to those found with the wt receptor (F.-Y. Zeng and J. Wess; unpublished results).

In the next step, we inserted three factor Xa (fXa) cleavage sites into the central portion of the i3 loop of the M3'(C3) mutant receptor. This modification had no significant effect on the ability of the resulting mutant receptor (referred to as M3'(C3)-Xa) to bind muscarinic ligands with high affinity and to efficiently couple to G proteins. Western blot analysis using membrane lysates prepared from M3'(C3)-Xa-transfected COS-7 cells showed that that M3'(C3)-Xa could be efficiently cleaved by the fXa protease (F.-Y. Zeng and J. Wess; unpublished results). Recently, we started to reintroduce pairs of cysteine residues (one N-terminal and one C-terminal of the fXa cleavage sites) into distinct intracellular regions of M3'(C3)-Xa, such as the i2 loop and the N- and C-terminal portions of the i3 domain. Application of mild oxidizing conditions to membrane lysates prepared from COS-7 cells transfected with these mutant constructs will enable adjacent cysteine residues to form disulfide bridges. Preliminary studies showed that cross-linked products can be identified via immunoprecipitation and Western blot analysis (following treatment with fXa). Systematic application of this approach should eventually lead to novel insights into the three-dimensional structure of the M3 muscarinic receptor. Moreover, this strategy should be generally applicable to other classes of GPCRs.

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MOLECULAR EVENTS ASSOCIATED WITH THE REGULATION OF SIGNALING BY M_2 MUSCARINIC RECEPTORS

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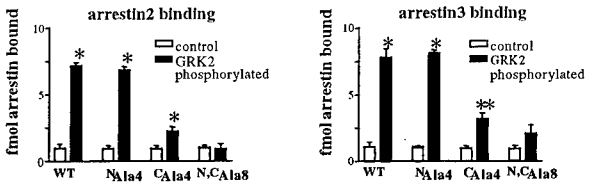
Summary

Multiple events are associated with the regulation of signaling by the M_2 muscarinic cholinergic receptors (mAChRs). Desensitization of the attenuation of adenylyl cyclase by the M_2 mAChRs appears to involve agonist-dependent phosphorylation of M_2 mAChRs by G-protein coupled receptor kinases (GRKs) that phosphorylate the receptors in a serine/threonine rich motif in the 3rd intracellular domain of the receptors. Mutation of residues 307-311 from TVSTS to AVAAA in this domain of the human M_2 mAChR results in a loss of receptor/G-protein uncoupling and a loss of arrestin binding. Agonist-induced sequestration of receptors away from their normal membrane environment is also regulated by agonist-induced phosphorylation of the M_2 mAChRs on the 3rd intracellular domain, but in HEK cells, the predominant pathway of internalization is not regulated by GRKs or arrestins. This pathway of internalization is not inhibited by a dominant negative dynamin, and does not appear to involve either clathrin coated pits or caveolae. The signaling of the M_2 mAChR to G-protein regulated inwardly rectifying K channels (GIRKs) can be modified by RGS proteins. In HEK cells, expression of RGS proteins leads to a constitutive activation of the channels through a mechanism that depends on $G\beta\gamma$. RGS proteins appear to increase the concentration of free $G\beta\gamma$ in addition to acting as GAPs. Thus multiple mechanisms acting at either the level of the M_2 mAChRs or the G-proteins can contribute to the regulation of signaling via the M_2 mAChRs.

Key Words: M_2 mAChRs, phosphorylation, arrestins, internalization, desensitization

G-protein coupled receptors signal through a variety of G-proteins and downstream effectors. These signaling events are regulated by a variety of mechanisms that help to "turn-off" the signals and return the cell to its resting, non-stimulated state. These "turn-off" mechanisms are very important in physiological situations, such as in a synapse where a relatively large concentration of released neurotransmitter can cause a rapid activation of events that control neuronal excitability in the postsynaptic neuron. By ensuring that the activated receptors also rapidly inactivate, the neuron is able to respond to the next wave of released neurotransmitter. Turning off signals is also important in pathological conditions. For example, in the chronic stage of Chagas disease, which is often



	WT	N_{Ala4}	C_{Ala4}	NC_{Ala8}
Desensitization of AC	Y	Y	N	N
Arrestin binding in vitro	Y	Y	N	N
Arrestin binding in situ	Y	Y	N	N
Arrestin on affinity	Y	Y	N	N

Fig. 1

Arrestin binding requires the presence of the serines and threonines in the motif TVSTS (residues 307-311) in the hM_2 mAChRs. The upper section of the figure summarizes results of *in vitro* binding studies with WT and mutant M_2 mAChRs and *in vitro* translated arrestins (3). Data in the lower box summarize results of analyses of arrestin interaction with WT and mutant hM_2 mAChRs in other assays (3,4,8).

associated with severe cardiomyopathy, circulating antibodies that have agonist-like activity at M_2 mAChRs are postulated to cause a chronic desensitization of the mAChRs and contribute to the functional blockade of these receptors that is observed in patients (1). Here we discuss molecular events that are associated with the regulation of signaling by M_2 mAChRs. Intricate mechanisms exist for controlling the signal transduction events at the level of the receptors and G-proteins. The rapid uncoupling of the M_2 mAChRs from G-proteins appears to be regulated by protein phosphorylation and arrestins (2-4). Internalization of M_2 mAChRs from the cell surface can occur via several pathways depending on the cellular machinery that is present. The predominant pathway of internalization of M_2 mAChRs in HEK293 cells does not involve the participation of GRKs or arrestins (2-4). In addition, the regulation of M_2 mAChR signaling to G-protein dependent inwardly rectifying potassium channels (GIRKs) involves the participation of the Regulators of G-protein Signaling (RGS proteins) that appear to act by multiple mechanisms at the level of G-proteins.

Agonist-dependent phosphorylation regulates receptor/G-protein uncoupling and arrestin binding

Several independent studies in native cardiac tissue as well as in heterologous expression systems has established that the M_2 mAChRs are phosphorylated on serine and threonine residues in response to agonist activation (5). Studies with a dominant negative allele of GRK2 demonstrated that expression of this construct reduced agonist-dependent phosphorylation by 50% in HEK cells and totally prevented the desensitization of the adenylyl cyclase response that was observed under control conditions (2). These results pointed out an important role for GRK mediated phosphorylation in the uncoupling of the M_2 mAChRs from the G_i proteins that couple the receptors to attenuation of adenylyl cyclase. Since the only cytoplasmic serine residues are on the 3rd intracellular domain, it was clear that this domain contains the phosphorylation sites. Indeed, we have identified a serine(S)/threonine(T) rich motif of residues 307-311 (TVSTS) as an important phosphorylation target that causes uncoupling of the human(h) M_2 mAChRs (4). With other receptors, namely the light receptor rhodopsin and the β_2-adrenergic receptor (β_2AR), GRK mediated phosphorylation

provides a signal for the binding of arrestins, which in turn are responsible for the uncoupling of the receptors from G-proteins (6,7). We asked if residues 307-311 were important for arrestin binding by using several different tests of receptor/arrestin interaction. Indeed, mutation of the S and T residues to alanines in this motif (in the C_{Ala4} and NC_{Ala8} mutants) led to a depression or abolition of arrestin binding *in vitro* to the mutant hM_2 mAChRs (Fig. 1), whereas mutation of the phosphorylation motif STSVS (residues 286-290) in the N_{Ala4} mutant was without effect (Fig. 1).

Similarly, studies of arrestin interaction with the WT and mutant M_2 mAChRs in other assay systems also demonstrated an inability of receptors mutated in the 307-311 motif (in either the C_{Ala4} or N,C_{Ala8} mutants) to interact with arrestins (Fig. 1) (3,4,8). These results are consistent with the concept that arrestin interaction with GRK-phosphorylated M_2 mAChRs may be the underlying cause of the uncoupling of the phosphorylated M_2 mAChRs from G-proteins and signalling pathways. However, definitive proof of this possibility awaits direct testing of the role of arrestins in the uncoupling process in intact cells.

Internalization of M_2 mAChRs in HEK cells proceeds through a different pathway than receptor uncoupling

An appealing finding from studies of the β_2AR is that the binding of arrestins to GRK phosphorylated β_2AR facilitates their endocytosis by delivering these receptors to clathrin coated pits (9,10). The C-terminal domains of the non-visual arrestins 2 and 3 (but not visual arrestin1) bind to clathrin, thus facilitating the initiation of receptor endocytosis (10). Surprisingly, although the M_2 mAChRs bind arrestin (Fig. 1), we have found that the internalization of the M_2 mAChR is not affected by the M_2 mAChR mutations that result in a loss of arrestin binding (4) or by expression of the dominant negative GRK that disrupts receptor-G-protein uncoupling (2)(Fig. 2).

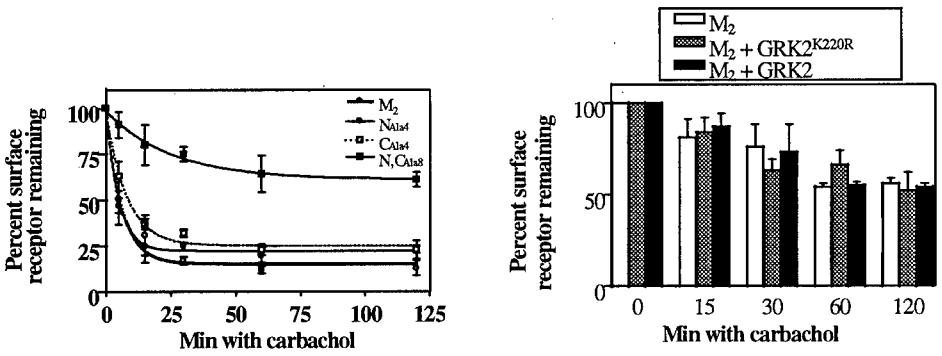


Fig. 2

Internalization is unaffected by receptor mutations and a dominant negative GRK2. Both panels show the percentage of cell surface M_2 mAChRs remaining as a function of time of exposure to carbachol (1 mM). Left panel: The C_{Ala4} mutant introduced above was found to internalize in a manner indistinguishable from the WT M_2 mAChR. As this mutant cannot bind arrestin (see Fig. 1), the observed internalization proceeds via an arrestin independent mechanism. Right panel: Expression of WT GRK2 or the dominant negative $GRK2^{K200R}$ did not enhance or inhibit the internalization of the WT M_2 mAChRs.

In other experiments the internalization of the M_2 mAChRs was found to be insensitive to overexpression of a dominant negative dynamin (3), a protein that has been implicated in the pinching off of clathrin coated pits (11) and caveolae (12,13). From these and other data, we concluded that the predominant pathway of internalization of the M_2 mAChRs in HEK cells occurs via a mechanism that does not involve arrestin, clathrin coated pits or caveolae. This unknown

pathway remains to be defined. Interestingly, internalization of the M_2 mAChR by this unknown pathway is facilitated by agonist-dependent phosphorylation of the M_2 mAChRs, since ablation of all phosphorylation in the NC_{Ala8} mutant lead to a slower rate and smaller extent of internalization (4) (see Fig. 2, left panel). It is likely that multiple pathways of internalization participate in the internalization of GPRs.

Signaling of the M_2 mAChR to inwardly rectifying potassium channels is modulated by RGS proteins

In addition to the receptor-dependent mechanisms of regulation of M_2 mAChR signaling that were introduced above, there are also other mechanisms that regulate signaling that act at the level of the G-proteins. Indeed, the RGS proteins have been shown to be GAPs (GTPase activating proteins) that accelerate the G-protein GTPase reactions, thus accelerating the turn off reactions and inducing desensitization in a wide variety of systems (14-17). The M_2 mAChRs activate GIRKs through a mechanism that involves release of $G_{\beta\gamma}$ subunits from G_i proteins and the direct activation of the channels by the $G_{\beta\gamma}$ subunits (18-20). When GIRKs were expressed in heterologous expression systems, their kinetics of deactivation following agonist treatment were observed to be much slower than in native systems (21,22). However, expression of the RGS proteins significantly accelerated the deactivation, suggesting that the RGS proteins were acting as GAPs in intact cells (21,22). However, a curious finding was that the peak currents were not reduced upon expression of the RGS proteins, and the kinetics of activation were accelerated (21,22). Neither of these effects were expected if the RGS proteins were solely acting as GAPs (15).

We assessed the effects of RGS3 and RGS4 on the activity of GIRKs 1 + 4 expressed in HEK293 cells. In addition to the effects on kinetics of activation and deactivation that were previously observed by others (21, 22), we observed that expression of the RGS proteins led to an increase in the "basal" activity of the channels. By using barium to block currents through the channels, we observed that ~50% of the total channel activity was activated in the absence of agonist in cells expressing RGS4 (or RGS3). In contrast, in control cells, less than 20% of the channels were active in the absence of agonist. The effects of RGS proteins to activate currents in the absence of agonist were not blocked by pertussis toxin, suggesting that the effects were not due to promotion of the coupling of M_2 mAChRs, Gi and GIRKs. However, the effects of RGS proteins to activate the GIRKs were totally suppressed by the expression of a $G_{\beta\gamma}$ binding protein, CD8-GRK2-CT, which had previously been demonstrated to act as a "sink" for $G_{\beta\gamma}$ and inhibit $G_{\beta\gamma}$-dependent signaling events (23). Thus, it appears that the RGS proteins activated the GIRKs by causing an increase in free $G_{\beta\gamma}$. It had previously been suggested that RGS proteins bind with high affinity to the transition state of the G_α subunits and accelerate the GTPase reaction (24,25). We speculate that the RGS proteins can bind also to other conformations of the G_α subunits, and thus liberate $G_{\beta\gamma}$ subunits, as the crystal structure of RGS suggests that simultaneous binding of RGS and $G\beta\gamma$ to G_α subunits would be unlikely (24). The data presented suggest that RGS proteins can act negatively to speed up the turn off of G-proteins, and additionally can act to enhance signaling by increasing the availability of $G_{\beta\gamma}$ subunits to their downstream effectors. It is likely that the net effects of RGS proteins will reflect the ability of the RGS proteins to interact with the different conformations of the G proteins. The effects of RGS proteins to increase availability of the $G_{\beta\gamma}$ subunits can at least partly explain why the peak currents attributable to GIRKs were not depressed by RGS expression (15,21,22). Future studies will reveal if our hypothesis is correct and if RGS proteins have other actions than to act as GAPs.

In summary, the regulation of signaling through the M_2 mAChRs involves events that modify both the ability of the M_2 mAChRs to interact with G-proteins and endocytic machinery. Future studies

will seek to determine if arrestins participate in the uncoupling of the M_2 mAChRs from G-proteins and to identify the non-arrestin dependent pathway of internalization. If arrestins do bind to the receptors to cause uncoupling from G-proteins, it will be of interest to determine why the arrestins do not also deliver the receptors to endocytic machinery. In addition, further studies will define how RGS proteins can negatively and positively modulate signalling by acting at the level of G proteins.

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THE INFLUENCE OF THE CELLULAR CONTEXT ON RECEPTOR FUNCTION: A NECESSARY CONSIDERATION FOR PHYSIOLOGIC INTERPRETATIONS OF RECEPTOR EXPRESSION STUDIES

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Summary

The cell model studied has a fundamental influence on the function and regulation of G protein linked receptors. These cell-dependent effects are illustrated in the current communication focusing on M_3 muscarinic, CCK and GRP receptors. Receptors interact with multiple cellular mechanisms. The most obvious are those involved in coupling to signaling mechanisms such as G proteins. Receptors are themselves phosphorylated and dephosphorylated by cellular kinases and phosphatases. Receptors may sequester, internalize, down-regulate and recycle via interactions with a number of separate cellular mechanisms. When the number and complexity of interactions between the cell and the receptor are taken into account it is not surprising that the cell model has a primary influence on receptor function and regulation. The implications of the importance of the cell model in receptor function for studies aimed at answering physiologic questions are discussed.

Key Words: desensitization, G proteins, G protein-coupled receptors, receptor expression studies

Much has been written concerning the influence of receptors on cell function. Much less discussed is the reverse relationship, the influence of the cellular context on receptor function and regulation. In recent years the ability to molecularly manipulate receptors and express them in novel cell types has lead to many new insights into the relationship between receptors and cells. We will discuss the impact of these observations on approaches to physiologic problems. We will focus on three receptors that function to regulate the pancreatic acinar cell, the M_3 muscarinic acetylcholine (m3ACh), cholecystokinin A (CCK_A), and gastrin releasing peptide (GRP) receptors.

The cell context affects receptor coupling

M_3 Ach, CCKA, and GRP receptors couple to the activation of the Gq family of guanine nucleotide binding proteins (G proteins). Activation of this family of G proteins increases phospholipase C β activity and accelerates the rate of phosphatidylinositol-(4,5)-bisphosphate (PIP2) hydrolysis (1). The breakdown of PIP2 generates inositol 1,4,5-trisphosphate (Ins(1,4,5)P3) and diacylglycerol (DAG). These last two molecules are key cellular messengers

leading to the release of Ca^{2+} from internal stores and the activation of protein kinase C respectively. The activation of this traditional phospholipase/Ca^{2+} pathway is key to the abilities of these receptors to stimulate secretion from the pancreatic acinar cell (2). Therefore, as might be expected, each of these receptors is equivalent in terms of stimulating pancreatic acinar cell secretion.

Coupling of these receptors to Ca^{2+} signaling has been observed in virtually every cell model in which they have been studied. For example, we found only minor differences in the coupling of CCK_A receptors in CHO cells compared with pancreatic acinar cells (3). This is likely because these receptors have a high affinity for Gq subunits, which are ubiquitously expressed (4). However, in a few cases some important differences in receptor coupling to major signaling pathways have been observed. An extreme example of cell-type differences in coupling comes from studies showing that wild-type GRP receptors transfected into the NCM460 colon cancer cell line appear to be constitutively active (5). However, this seems to be the exception rather than the rule and for the most part coupling of receptors to their preferred signaling pathways occurs similarly in most cell models.

M_3 Ach, CCK_A and GRP receptors are also able to activate a variety of other, non-Gq, signaling pathways. There are three mechanisms whereby the coupling to multiple signaling pathways may occur; promiscuous G protein coupling, separate effects of α and $\beta\gamma$ subunits, and signal cross-talk. Like other G-protein linked M_3 Ach, CCK_A and GRP receptors are somewhat promiscuous and can form interactions with multiple G proteins and effectors. For example, in native pancreatic acinar cells CCK_A receptors couple to the generation of cAMP (6). This is thought to be due to a weak interaction with Gs. An alternative explanation for this observation would be that it is due to some form of "cross-talk" between signaling pathways. Cross-talk describes the situation when one signaling pathway triggers the activation of a separate signaling pathway. For example, adenylyl cyclase exists in multiple isoforms, some of which can be activated by Ca^{2+} (7). Therefore, in cells in which exist these isoforms of adenylyl cyclase increases in intracellular Ca^{2+} concentration will indirectly increase cAMP levels. However, cross-talk is not thought to explain the effects of CCK_A receptor activation in pancreatic acinar cells as neither increasing Ca^{2+} concentration nor activating protein kinase C increases intracellular cAMP in these cells. Interestingly, neither M_3 Ach nor GRP receptors on pancreatic acinar cells appear to couple to activation of Gs. Yet, either receptor expressed in CHO cells readily couples to the generation of cAMP.

Other signaling pathways activated by these receptors involve tyrosine phosphorylation. For example, activation of the CCK_A receptor on pancreatic acinar cells has been shown to stimulate MAP kinases including the ERKs (8) and JNKs (9;10). Both M_3 Ach and GRP receptors have also been found to activate ERKs in pancreatic acinar cells (8). However, M_3 Ach and GRP receptors are poor activators of JNKs in pancreatic acinar cells (9;10). In contrast, M_3 Ach receptors strongly activate JNKs in other cell models (11). A dramatic example of cell-type dependent differences in coupling to these pathways has been reported for CCK_B receptors. CCK_B receptors expressed in the parietal cells of *Mastomys natalensis* are fully coupled to generation of inositol phosphates but do not activate MAP kinases (12). In contrast, CCK_B receptors on ECL cells from the same species while coupling to phospholipid hydrolysis with the same efficacy were shown to also activate the MAP kinases.

Different cells respond differently to receptor activation

Within multicellular organisms cells organize into tissues that specialize in carrying out specific functions. Clearly the activation of signaling pathways generates different biological responses depending upon the differentiated functions of the particular cell. Thus, activation of M_3

muscarinic receptors will cause the contraction of smooth muscle cells while stimulating secretion from pancreatic acinar cells. Generally it is not possible to study these types of differentiated functions in tissue culture cell models because cultured cells tend to be relatively undifferentiated. However, some cell functions such as growth and mitogenesis are universal and the effects of receptor activation on these functions can be evaluated in any cell.

Activation of Gq coupled receptors has been shown to lead to increased cellular growth rates in numerous cell types. Furthermore, when expressed in NIH3T3 cells M_3 Ach receptors can act in an agonist dependent manner to cause cellular transformation (13). Similar results have been reported for other Gq linked receptors in this cell type (14). However, in other cell types, for example CHO cells, activation of M_3 Ach receptors causes growth inhibition and suppresses the transformed phenotype (15). Likewise, activation of CCK_A receptors inhibits growth and transformation of CHO cells (16). Growth inhibition by Gq coupled receptors has also been reported in some other cell types (17).

The explanation for the observance of opposite effects on growth in different cells is not currently clear. The obvious possibilities are that the receptors may couple to different signals in the different cells (see above) or the cells may respond differently to the same signals depending upon other aspects of the cell context. Both these possibilities are likely true under some circumstances. However, because the major signaling pathways are most likely to have predominant effects and coupling to these major pathways occurs in virtually all cells, it is most likely that differences other than receptor coupling explain the differences observed in growth regulation.

The cell context affects receptor trafficking and desensitization

The effects of receptor activation are determined not only by the specific signaling pathways that are activated, but also by the duration of their activation. Intensity and duration of receptor signaling are influenced by numerous factors. The importance of signal duration depends upon the cellular response activated. For responses of short-duration, such as secretion, receptor activation for seconds to minutes results in full manifestation and only rapid desensitization processes are able to influence the response to an initial stimulation. For responses of long-duration, such as cell growth, all desensitization mechanisms could be involved in determining the ultimate biological response.

We found that M_3 Ach receptor desensitization limited the growth inhibitory effects of receptor activation observed in CHO cells (15). Activation of the M_3 Ach receptor in the CHO cell lead to a transient inhibition of DNA synthesis and cell division. However, this inhibitory effect declined over-time such that the cells resumed normal growth rates within 24-48 hours of continuous agonist exposure. In contrast, a mutant desensitization deficient M_3 Ach receptor caused a prolonged inhibition (15).

Receptor sequestration is an aspect of receptor regulation that varies widely in different cell types. Sequestration is a relatively rapid process of receptor movement away from the cell surface to a compartment that is inaccessible to hydrophilic ligands. It is unclear whether sequestration represents a desensitization or a resensitization mechanism, and this may vary between receptors. Sequestration is most easily monitored with receptors for which both hydrophilic and hydrophobic ligands exist. In this case, sequestration is manifest as a decrease in binding of the hydrophilic ligand without a corresponding decrease in binding of the hydrophobic ligand. Using this type of assay it has been observed that M_3 ACh receptors sequester readily in a variety of cell types including native pancreatic acinar cells (18) and

human embryonic kidney (HEK) 293 cells (19). However, M_3 Ach receptors do not sequester in CHO cells (20).

Receptor down-regulation also occurs to different extents in different cell types. Receptor down-regulation is the loss of receptors due to receptor degradation. Generally receptors are thought to be endocytosed upon agonist occupation and the internalized receptors may be either recycled to the plasma membrane, or targeted to lysosomes for degradation. M_3 ACh receptors down-regulate significantly in native pancreatic acinar cells (18;21) and in CHO cells (20), but not in HEK 293 cells (19). Down-regulation is a component of receptor desensitization. The relative importance of this component of desensitization depends upon the response in question and in the cell type. In CHO cells, the M_3 Ach receptor shows very little rapid desensitization, and in this cell type down-regulation appears to play an important role in desensitization (15;20). In contrast, in HEK 293 cells rapid desensitization is observed and down-regulation does not seem to be important (19).

Receptor internalization is likely important for both sequestration and down-regulation. In fibroblastic cells most G protein coupled receptors are internalized via a clathrin mediated mechanism. This process has been well described morphologically (22). The formation of clathrin-coated vesicles is a complex process and this pathway involves a number of proteins including actin and dynamin. G protein $\beta\gamma$ subunits may play an important role in this pathway (23). Receptors may also internalize via non-clathrin mediated pathways. One potential non-clathrin pathway involves caveolae. Caveolae appear to be involved in receptor internalization in some cells (24;25) but not others (26). Dynamin may also play a role in caveolae formation (27). With so many complex mechanisms potentially involved in receptor internalization it is not surprising that receptors are internalized differently by different cells. For example, CCK_A receptors internalize into a cytoplasmic domain in both clathrin-dependent and clathrin independent modes in CHO cells (28). Internalization of CCK_A receptors into NIH3T3 cells also appears to be largely mediated via a clathrin-mediated pathway (29). In contrast, CCK_A receptors do not enter the cytoplasm of native pancreatic acinar cells but instead are confined to specific plasma membrane domains (30). Receptor internalization is generally an agonist-mediated event. However, some constitutive internalization of receptor also occurs. The relative level of agonist-independent internalization of the CCK_A receptor has been shown to be dependent on the cell type. Internalization of the CCK_A receptor was highly dependent on agonist occupation in HeLa, Cos-1, and CHO cells, but not in NIH3T3 cells (29).

Receptor phosphorylation is an important mechanism involved in receptor regulation. Phosphorylation of the receptor by receptor kinases may lead to uncoupling from G protein activation and may also be involved in internalization, down-regulation or recycling of receptors (31). A number of cellular kinases are capable of phosphorylating receptors, including a special class of G protein-coupled receptor kinases (32). Receptor phosphorylation varies depending upon the cell type. For example, phosphopeptide mapping has shown that CCK_A receptor phosphorylation in native pancreatic acinar cells is different than that observed in CHO cells (33). This may be due to differences in the types or levels of various kinases. Variations in receptor kinases have been suggested to explain the observed variations in cellular desensitization of the β_2-adrenergic receptor in the lung (34).

Cellular variations in receptor numbers and components of receptor function/regulation likely explain different observations in different cells.

Variations in the quantitative relationships between receptors and cellular molecules with which they interact likely explain cellular variations in receptor function and regulation. One major source of variation is the level of receptor expression. Several studies have investigated the

relationship between receptor levels and receptor function and regulation (35;36). At higher receptor expression levels coupling to alternative signaling pathways is often observed (37;38). Few studies have been conducted on the effects of receptor number on receptor regulation but effects on receptor down-regulation have been reported (35).

The second source of variation is differences in the identities and levels of the various proteins that interact with receptors. Receptors interact with a large number of proteins in an agonist-regulated manner. G proteins, both α subunits and $\beta\gamma$ subunits are involved in activating cellular signaling pathways. G protein-coupled receptor kinases and other cellular kinases interact with and phosphorylate receptors. Receptors can interact with arrestin-like molecules. Receptors are dephosphorylated by a number of phosphatases. Receptors are internalized by clathrin-dependent and –independent pathways. When the large number of cellular proteins that interact with receptors are taken into account it is not difficult to understand the importance of the cellular context on receptor function.

Impact of cellular differences on the understanding of receptor regulation under physiologic circumstances

From the above discussion it is clear that what is observed experimentally in terms of receptor function and regulation depends largely upon the cell model in which the receptor is expressed. This means that results reported in one cell model cannot automatically be generalized to other cell models. Obviously then, experimenters should use great care in selecting cell models. This is particularly important when the questions being investigated are physiologic rather than biochemical in nature. To understand how receptors affect a particular physiologic function and how they are regulated in a physiologic setting requires careful attention to the specific characteristics of the cells. Recently it has become possible to rapidly generate data on receptor structure and function. The time between the description of a phenomenon and the identification of structural elements within the receptor that are involved has decreased dramatically. However, it remains critically important to determine the physiological context within which these phenomena occur.

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MOLECULAR ANALYSIS OF THE REGULATION OF MUSCARINIC RECEPTOR EXPRESSION AND FUNCTION

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Summary

We have investigated the molecular mechanisms involved in the regulation of muscarinic acetylcholine receptor gene expression and localization and generated knockout mice to study the role of the M_1 muscarinic receptor in vivo. We have used the MDCK cell system to demonstrate that different subtypes of mAChR can be targeted to different regions of polarized cells. We have also examined the developmental regulation of mAChR expression in the chick retina. Early in development, the M_4 receptor is the predominant mAChR while the levels of the M_2 and M_3 receptors increase later in development. The level of M_2 receptor is also initially very low in retinal cultures and undergoes a dramatic increase over several days in vitro. The level of M_2 receptor can be increased by a potentially novel, developmentally regulated, secreted factor produced by retinal cells. The promoter for the chick M_2 receptor gene has been isolated and shown to contain a site for GATA-family transcription factors which is required for high level cardiac expression. The M_2 promoter also contains sites which mediate induction of transcription in neural cells by neurally active cytokines. We have generated knockout mice lacking the M_1 receptor and shown that these mice do not exhibit pilocarpine-induced seizures and muscarinic agonist-induced suppression of the M-current potassium channel in sympathetic neurons.

Key Words: muscarinic receptor, receptor localization, retina, M_2 promoter, knockout mice

Our laboratory has been interested in identifying the mechanisms which regulate the expression and function of the muscarinic acetylcholine receptors (mAChR) as part of the overall goal of determining the molecular and cellular bases for the regulation of cholinergic transmission. In this article we will describe studies on the subcellular localization of the mAChR, analyses of the regulation of mAChR gene expression by developmentally regulated factors and by cytokines, and the analysis of the function of the M_1 mAChR in the nervous system in vivo.

Differential localization of mAChR subtypes

A great deal of research has been directed at determining the molecular basis responsible for the selectivity of coupling of a specific mAChR subtype to the G-proteins with which it interacts. The functional responsiveness of a given receptor can depend not only on which G-proteins it can activate but also on where in a cell that receptor is localized. There have been a number of reports suggesting the differential subcellular localization of specific mAChR subtypes. Thus, both the functional responses and numbers of mAChR are asymmetrically distributed in *Xenopus* oocytes; a combination of both molecular biological and

pharmacological experiments demonstrate that the endogenous M_1 and M_3 receptors in the oocyte are differentially distributed in the cell membrane (1, 2). The mAChR-mediated release of intracellular calcium is initiated at a discrete region of pancreatic acinar cells (3) and lacrimal acinar cells (4), and muscarinic agonists evoke electrophysiological changes in lingual epithelial cells only when applied to the serosal but not mucosal membrane (5). mAChR are also thought to be differentially distributed in neurons. There is considerable pharmacological evidence that the M_1 receptor is most commonly (but not uniquely) postsynaptic and that the M_2 receptor is presynaptic (references in ref. 6). Immunocytochemical localization using subtype specific antibodies is consistent with this distribution. However, it is clear from both pharmacological and immunocytochemical experiments that a given receptor subtype may be sorted to different subcellular regions in different types of neurons (7,8).

The mechanisms for intracellular sorting of proteins have been best investigated in polarized epithelial cells. There are multiple mechanisms for the generation of protein asymmetry, including direct sorting to apical or basolateral membranes, transcytosis of proteins from the basolateral to the apical surface, and transport to both apical and basolateral domains but with selective stabilization of the protein at the basolateral membrane (9, 10). Madin Darby canine kidney (MDCK) cells have been used recently to examine the mechanisms involved in the differential localization of α_2-adrenergic receptors subtypes (10). We have found that MDCK cells express significant levels of endogenous mAChR which are differentially distributed and couple asymmetrically to signal transduction pathways between apical and basolateral domains. When grown on Transwell filters, the level of [^{3}H]N-methylscopolamine binding sites on the basolateral membrane is 2-3 times that on the apical membrane. Interestingly, only the apical receptors produce a detectable functional response: incubation of the apical cell surface with carbachol causes robust inhibition of forskolin-stimulated cAMP formation but does not stimulate phospholipase C activity, while incubation of the basolateral cell surface with carbachol affects neither intracellular cAMP levels nor phospholipase C activity. We have also found that exogenously expressed mAChR in transfected MDCK cells are also localized in different regions of MDCK cells: when transfected into MDCK cells, the M_2 receptor was localized to the apical domain, the M_3 receptor was localized to the basolateral domain, and the M_1, M_4, and M_5 receptors were not polarized. These results suggest that the MDCK cell system will be a useful one to determine the molecular mechanisms and sorting signals responsible for the differential localization of mAChR subtypes in polarized cells.

Regulation of retinal mAChR gene expression during embryonic development

Biochemical studies have shown that there are changes in the expression of mAChR in the chick retina during embryonic development (11). Affinity alkylation and SDS gel electrophoresis of retinal mAChR early in development identified a large molecular weight species (86 kDa) as the predominant form; later in development the main form of mAChR was a lower molecular weight species (72 kDa). We used immunoprecipitation and immunoblot analyses and solution hybridization to identify the subtypes of mAChR expressed in the chick retina and to determine if there were changes in their expression during development (12). Early in development, the main subtype is the M_4 receptor, while the expression of the M_3 receptor increased somewhat and the expression of the M_2 receptor increased dramatically during the second week of embryonic development. Immunoblot analyses demonstrated that the apparent molecular weight of the M_2 receptor was significantly less than that of the M_3 and M_4 receptors. The change in molecular weight reported in the early biochemical studies could thus be explained by the developmentally regulated increase in expression of the M_2 receptor.

Biochemical studies also reported that a similar shift in molecular weight occurred in dissociated chick retinal cultures. It was suggested that this shift in apparent molecular weights was due to the action of a developmentally regulated secreted factor (13). We therefore used immunoprecipitation, immunocytochemistry, and solution hybridization analyses to investigate the regulation of mAChR expression in dissociated retinal cell cultures (14). When retinal cultures are prepared from nine-day embryos, the level of M_2 receptor expression is very low, and increases significantly over days 2-4 in culture. Growth of retinal cells in conditioned

medium isolated from mature (i.e., cultured for ≥ 3 days) but not young cultures resulted in a large increase in the expression of M_2 protein and mRNA, without affecting the expression of the other mAChR subtypes. The secreted factor which is responsible for induction of M_2 receptor expression has been partially purified from serum-free medium and shown to be protease-sensitive with a molecular weight greater than 10,000. The M_2 -inducing factor stimulates expression of a luciferase reporter gene under the transcriptional control of the chick M_2-promoter in transiently transfected retinal neurons, demonstrating that the induction of M_2 expression results from increased gene transcription. Incubation of cultured retinal cells with a large number of known neurotrophic and growth factors did not increase the expression of the M_2 receptor. A novel developmentally regulated secreted factor may thus be responsible for the subtype-specific induction of M_2 receptor gene transcription in chick retina.

Isolation and functional analysis of the promoter for the chick M_2 receptor gene

We isolated the promoter for the chick M_2 receptor gene in order to identify the factors and mechanisms responsible for regulation of mAChR gene expression in both neural and cardiac cells. There is a long (>8 kb) intron in the 5' untranslated region of M_2 receptor gene, with over 320 basepairs between the intron-exon boundary and the 3'-most start site of transcription. Primer extension and RNase protection experiments demonstrated that the promoter region contains multiple transcriptional start sites that are used in both cardiac and neuronal cells. Transfection experiments using luciferase reporter genes driven by various regions of the M_2 promoter demonstrated that a 789 basepair (bp) fragment was sufficient for high level expression in primary cultures of chick heart cells, while 2 kilobasepair (kb) of promoter sequence was required for maximal expression in SN56 septal-neuroblastoma cells (15). The 789 bp region required for cardiac expression contained three consensus sites for GATA factors, a family of transcription factors which have been implicated in the expression of a variety of genes in both hematopoietic (GATA-1, -2, and –3) and cardiac (GATA-4, -5, and –6) cells. Cotransfection of cloned chick GATA-4, -5, or –6 into JEG-3 human choriocarcinoma cells resulted in a dramatic induction of M_2-luciferase reporter gene expression. Mutagenesis experiments showed that only the 3′ most GATA site was required for this induction. This site was also required for maximal expression in cardiac cells. Electrophoretic mobility shift assays demonstrated that the 3′ GATA site, but not the other two sites, exhibited specific binding both for a protein present in chick heart nuclear extracts and for recombinant chick GATA-4, -5, and –6. These experiments are the first to identify a protein required for maximal basal expression of a mAChR gene and the first to identify a factor required for mAChR expression in the heart (16).

Treatment of SN56 or IMR-32 neuroblastoma cells with the cytokines leukemia inhibitory factor (LIF) or ciliary neurotrophic factor (CNTF) increased mAChR number and increased the expression of the M_2 promoter reporter genes. Initial mapping studies indicated that the same 2 kb region of the promoter required for high level neural expression also conferred sensitivity to cytokines (15). Preliminary results suggest that there are multiple elements in this region which are each sufficient to mediate induction of M_2 transcription.

The LIF and CNTF receptors contain two transmembrane polypeptides, termed the low affinity LIF receptor and gp130, respectively, which mediate cytokine signaling. Transfection of SN56 or IMR-32 neuroblastoma cells with chimeric receptors containing the intracellular domains of these two polypeptides demonstrated that homodimers containing gp130 could induce M_2 gene transcription, while homodimers containing the low affinity LIF receptor were ineffective. We used deletion analysis and site-directed mutagenesis to identify two tyrosine residues near the carboxy-terminus of gp130 which were required for cytokine induction of M_2 receptor gene transcription (17). These two tyrosines have been previously shown to serve as docking sites for the transcription factors Stat1 and Stat3, suggesting that one or both of these factors may be required for M_2 gene induction.

Functional analysis of the M_1 receptor in knockout mice

Muscarinic receptors have been implicated in a wide range of functions in the brain. We have used gene targeting by homologous recombination in embryonic stem cells to generate mice

lacking the M_1 receptor (18). Embryonic stem cell clones in which the mutant gene was incorporated into the genome by homologous recombination were used to obtain M_1 knockout mice. Homozygous M_1 mutant mice were born from crosses of heterozygote mice with the predicted ratio of 1:4, and were indistinguishable from wildtype mice in body weight, longevity, fertility, and overt behavior.

The number of mAChR binding sites in the forebrains of knockout was approximately half of that in wildtype mice, while the level of mAChR sites in the cerebellum was similar in wildtype and knockouts. Immunoprecipitation analyses demonstrated that there was no detectable expression of M_1 receptor in the brains of knockout mice, while the levels of M_2, M_3, and M_4 receptors were not affected. Immunocytochemical analyses showed that there were also no significant differences in the morphology of the brain and in the pattern or levels of expression of the other receptor subtypes.

Incubation of sympathetic neurons derived from wildtype mice with the muscarinic agonist oxotremorine-M causes pronounced suppression of the M-current potassium channel. The M-current in sympathetic neurons from knockout mice was insensitive to oxotremorine-M. In contrast, treatment with angiotensin II resulted in suppression of the M-current in both wildtype and mutant neurons. Thus, the M_1 receptor is the only muscarinic receptor subtype which suppresses M-current potassium channel activity in sympathetic neurons.

Systemic administration of the muscarinic agonist pilocarpine results in tonic-clonic seizures similar to those exhibited by patients with temporal lobe epilepsy. Mice homozygous for the mutant M_1 receptor do not exhibit seizures following the administration of doses of pilocarpine which cause multiple tonic-clonic seizures in wildtype mice. While heterozygous mutant mice express half the normal level of M_1 receptor, they are nearly as resistant as homozygous mutant mice to pilocarpine-induced seizures. Both wildtype and mutant mice exhibit seizures following administration of kainic acid. These results show that the M_1 receptor is required for the initiation of seizures in the pilocarpine model of epilepsy.

Because the M_1 receptor is expressed at high levels in the striatum, we have examined the effects of M_1 receptor gene disruption on striatal function. Preliminary results suggest that the M_1 knockout mice exhibit alterations in haloperidol-induced catalepsy and amphetamine-induced locomotor activity.

The M_1 mutant mice should be useful in determining the pathways involved in seizure initiation and in the other roles of the M_1 receptor in the nervous system.

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DEVELOPMENT OF MUSCARINIC RECEPTORS AND REGULATION OF SECRETORY RESPONSIVENESS IN RODENT SWEAT GLANDS

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Summary

Sweat glands are innervated by sympathetic neurons which undergo a change in transmitter phenotype from noradrenergic to cholinergic during development. As soon as the glands begin to differentiate, M3 muscarinic receptor mRNA and binding sites are detectable. Receptor expression appears in the absence of innervation and is maintained after denervation. While receptor expression is not regulated by innervation, secretory responsiveness is. Muscarinic blockade during development or in adult animals results in the loss of responsiveness and its reappearance requires several days. Cholinergic muscarinic activation is most likely to regulate one or more steps in the signalling cascade that are downstream of calcium mobilization. The anterograde regulation of sweat gland responsiveness is one facet of the reciprocal interactions are required to establish a functional synapse in this system.

Key Words: sympathetic innervation, sweat gland secretion, transmitter plasticity

Rat sweat glands provide an interesting system to study the development and regulation of mAchR. Concentrated in rat footpads, sweat glands are innervated by cholinergic sympathetic neurons. Homogenates of footpad tissue contain high levels of choline acetyltransferase (ChAT) activity, the synthetic enzyme for acetylcholine, and immunoreactivities for ChAT and the vesicular acetylcholine transporter (VAChT) are expressed at high levels in the gland innervation (1,2). Consistent with this neurochemical data, sweat secretion exhibits a muscarinic pharmacology in both adult and developing rodents; nerve-evoked sweating is blocked by muscarinic antagonists and secretion is elicited by local or systemic injection of muscarinic agonists (3-6). The muscarinic receptor expressed by the glands is the M3 glandular receptor and both the affinity and concentration of these sites are comparable to those described for other peripheral tissues; using selective muscarinic antagonists, we and others have determined that the site exhibits high affinity for 4-DAMP, intermediate affinity for pirenzepine, and low affinity for AF DX-116 (7,8). Further characterization of receptor expression using pharmacological probes and in situ hybridization have demonstrated that the receptor corresponds to the m3 molecular subtype (7-9). The secretory tubules of rodent sweat glands contain two cell types, secretory cells and myoepithelial cells (10,11). Since both cell classes respond to muscarinic agonists, it seems likely that both express receptors although this has not been shown directly as it has for the two cell classes in the lacrimal gland (12). In addition to muscarinic receptors, rat sweat glands express α_1 and β_2 receptors. Adrenergic agonists, however, are relatively ineffective in

eliciting sweat secretion (4,6,13,14).

Although the mature gland innervation is cholinergic, during development the innervation initially expresses noradrenergic properties (11,15,16). Sweat glands and their innervation develop postnatally (11). On postnatal day (P) 2, numerous shallow invaginations are evident in the epidermis of the developing footpads; these rapidly elongate and form presumptive secretory tubules by P7. By the middle of the second postnatal week, the forming gland has begun to coil and at P14 morphologically differentiated myoepithelial and secretory cells are present. On postnatal day 14 a minority of glands (< 1 %) secrete in response to both nerve stimulation and injection of muscarinic agonists but by the end of the third postnatal week most glands respond (4,6). At birth, when sympathetic axons first contact the developing sweat glands, they exhibit only catecholaminergic markers, including catecholamine fluorescence and immunoreactivity for the catecholamine synthetic enzymes, tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DbH). The cholinergic properties that characterize the mature innervation begin to appear at the end of the first postnatal week; VAChT is first evident at postnatal day 6 and ChAT activity at postnatal day 11 (1,17). During the third postnatal week, catecholamine stores disappear and TH and DβH immunoreactivities decrease but do not disappear.

One of the first signs of differentiation of the presumptive gland cells is the appearance of m3 message and muscarinic binding sites on P4 when division is actively occurring (18). During the second postnatal week the levels of mRNA encoding the m3 receptor and of muscarinic binding sites increases rapidly and reaches adult levels at P14. When the developmental time course of muscarinic receptor expression is compared with that of ChAT activity, it is clear that receptor expression precedes the appearance of cholinergic properties in the innervation. ChAT activity is not detectable until P11, it is less than 20% of adult levels on P14 and it continues to increase over the next several weeks (1). Similarly, VAChT is not detectable in the sweat gland innervation until P6 and it does not reach adult levels until P14 (1,16). Instead, at the time when muscarinic receptors are appearing, the glands are innervated by catecholaminergic axons (11). Since there are several different subtypes of muscarinic receptors, it was possible that receptor subtype expression could switch in sweat glands during development. Changes in muscarinic receptor expression have been described in the heart; initially high levels of chick m3 are detected in heart but these decrease as m2 increases (19). In developing sweat glands, however, only m3 receptor message was detected (18). Thus, the muscarinic cholinergic receptors expressed in this autonomic target tissue, at least, consist of a single molecular subtype which appears unchanged throughout development.

The normal pattern of innervation during development is not required to achieve the characteristic adult complement of muscarinic receptors (18). In rats treated with 6-OHDA at birth, sweat glands are never innervated by sympathetic axons (20) but they express the same receptor subtype defined by pharmacological and molecular criteria. Further, the concentration and affinity is also not significantly different from control, nor is the distribution of ligand binding sites analyzed at the light microscopic level. Thus, the early appearance of muscarinic receptors in the developing glands suggests that their initial expression is part of an intrinsic program of differentiation of the secretory tubule that is triggered by interactions between the epidermis and dermis. Further, denervation of adult rat sweat glands does not significantly change either the concentration or affinity of muscarinic receptors nor does it detectably alter the molecular subtype expressed (8). Thus, cholinergic innervation does not regulate muscarinic receptor expression. This finding contrasts with the finding that one of the adrenergic receptors expressed

by developing sweat glands, α_1, is downregulated during development and that this depends on innervation (14).

Development of sweat glands in the absence of sympathetic innervation leads to nonresponsive sweat glands in the adult and denervation of glands in adult animals leads to a loss of responsiveness (4). Neither treatment, however, changes the concentration or distribution of muscarinic receptors, nor is the molecular subtype expressed by the gland altered (8,18). Stimulation of muscarinic receptors of normal adult glands increases phosphoinositide turnover. Similarly, glands that are nonresponsive due to development in the absence of sympathetic innervation or to acute denervation after section of the sciatic nerve exhibited normal levels of muscarinic-mediated phosphoinositide turnover. These observations suggest that the sympathetic innervation provides an important retrograde signal that induces responsiveness during development and maintains it in the adult.

A logical candidate for mediating the anterograde effects of the innervation on secretory responsiveness is acetylcholine (21). As summarized above, muscarinic receptors are present as soon as the glands begin to differentiate. When the non-selective antagonist, atropine, or the selective antagonist, 4-DAMP, was administered to rat pups, the normal appearance of secretory responsiveness was prevented. In adult rats, atropine treatment leads to the disappearance of responsiveness. Following the withdrawal of atropine, however, secretory function develops in pups and returns in adults. The effects of the chronic blockade of muscarinic transmission with atropine on secretory function do not appear to reflect either gross perturbation of gland development or the presence of residual atropine at the time of assay. In addition, replacement with pilocarpine of the synaptic cholinergic activation lost after glands are denervated by sciatic nerve transection largely prevents the loss of function that normally occurs. The simplest interpretation of our data is that the muscarinic receptors blocked by atropine and activated by pilocarpine to alter secretory responsiveness are located on the glands, themselves. Thus, these results provide evidence that, in addition to its role as a traditional neurotransmitter in eliciting secretion from rat sweat glands, acetylcholine is responsible for the long term regulation of secretory function.

Since muscarinic receptor number and type is not regulated by innervation during development or denervation in the adult, activation of muscarinic receptors on the gland cells must regulate one or more steps in the downstream signalling cascade. The signal transduction pathway(s) responsible for coupling muscarinic ligand binding to secretion in rodent sweat glands has not been fully elucidated. Thus far, sweat secretion in rats and mice has been shown to be calcium dependent and sweat consists of a high potassium and chloride effluent (6,22). Studies performed on other exocrine gland cells, principally lacrimal cells, suggest that secretion is stimulated *via* the phosphotidylinositol (PI) pathway. In lacrimal cells the generation of IP_3 promotes the release of intracellular Ca^{2+} which activates potassium and chloride ion channels (23). In addition, the coupling of muscarinic receptors to secretion has been extensively investigated in the parotid gland, where it is also thought to be regulated through the PI pathway (24,25). More recent studies indicate that the m3 receptor in parotid gland is coupled not only to the stimulation of calcium mobilization but also the inhibition of cAMP accumulation (26). It seems likely that a similar mechanism occurs in rat sweat glands where the m3 molecular subtype, which has been shown previously to be preferentially coupled to PI turnover, is expressed (27,28).

There are several points in the signal transduction and secretory pathway at which the cholinergic

innervation could control responsiveness. One possibility is that innervation regulates the expression of the appropriate muscarinic receptor subtype. Our findings that the molecular subtype of muscarinic receptor and the levels of mRNA develop normally in the absence of innervation and remain unchanged following denervation makes this unlikely (8,18). A second explanation for functional nonresponsiveness is that a functional receptor is present but uncoupled from the secretory pathway. This does not seem to the case since the stimulation of phosphoinositol hydrolysis by muscarinic agonists is indistinguishable in uninnervated and denervated glands from that observed in responsive glands (8,18). Thus, the step in the pathway linking ligand binding to sweat secretion that is regulated by acetylcholine is distal to second messenger generation, either the ion channel(s) themselves or modulation of their function. Consistent with the possibility, when developing or adult rats are treated with muscarinic antagonists, several days are required for normal function to be established or reestablished following antagonist removal (21). In addition, more than 3 days of atropine treatment are required for loss of function. The length of time necessary suggests that the changes in responsiveness, onset, recovery and loss, are not due simply to the post-translational modification of pre-existing proteins or cycling of pre-existing proteins into or from an intracellular pool but rather to changes in gene expression, possibly genes coding for ion channels, in response to activation of muscarinic receptors. That the repertoire of ion channels in autonomic target tissues can be regulated by neurotransmitters is suggested by analysis of sodium channel gating in heart cells: the normal developmental change that occurs in the properties of cardiac voltage-gated sodium channels is induced in vitro by co-culture with sympathetic neurons and this induction is blocked by adrenergic antagonists (29).

The anterograde signalling mediated by muscarinic receptor activation that is responsible for inducing and maintaining secretory responsiveness is one facet of the reciprocal signalling required for establishing a functional synapse between sympathetic neurons and sweat glands. The change in neurotransmitter phenotype from noradrenergic to cholinergic that occurs during development is retrogradely specified by interactions with the sweat glands. The results of transplantation experiments indicate that sweat glands can induce changes in the transmitter phenotype of sympathetic neurons (30). Not only can sweat glands induce cholinergic properties in sympathetic neurons but interactions with sweat glands are required for the developmental changes that normally take place in sweat gland neurons. Analysis of *tabby* mutant mice, in which sweat glands fail to form, provides evidence for a requirement for target instruction. Sweat glands are not required for sympathetic axons to pathfind accurately to the presumptive target field in the footpads (31). While the gland-targeted axons reach the appropriate footpad region in the absence of forming glands and linger in the target area for approximately two weeks, they do not undergo changes in neurotransmitter phenotype (16). The retrograde specification of a new transmitter phenotype in sweat gland neurons is mediated by a cholinergic inducing factor secreted by the glands. The cholinergic inducing factor responsible for altering the transmitter properties of sweat gland neurons appears to be a member of the neuropoietic cytokine family. It is not, however, leukemia inhibitory factor, ciliary neurotrophic factor or cardiotrophin-1, but rather appears to be a novel member of the family (32). In culture, production of this factor is dependent upon adrenergic innervation (33,34). Analysis of catecholamine deficient mice confirms a contribution of adrenergic innervation to the change in transmitter properties but suggest that adrenergic innervation is not required in vivo (35).

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CONTRACTILE ROLE OF M_2 AND M_3 MUSCARINIC RECEPTORS IN GASTROINTESTINAL SMOOTH MUSCLE

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Summary

Muscarinic agonists elicit contraction through M_3 receptors in most isolated preparations of gastrointestinal smooth muscle, and not surprisingly, several investigators have identified M_3 receptors in smooth muscle using biochemical, immunological and molecular biological methods. However, these studies have also shown that the M_2 receptor outnumbers the M_3 by a factor of about four in most instances. In smooth muscle, M_3 receptors mediate phosphoinositide hydrolysis and Ca^{2+} mobilization, whereas M_2 receptors mediate an inhibition of cAMP accumulation. The inhibitory effect of the M_2 receptor on cAMP levels suggests an indirect role for this receptor; namely, an inhibition of the relaxant action of cAMP-stimulating agents. Such a function has been rigorously demonstrated in an experimental paradigm where gastrointestinal smooth muscle is first incubated with 4-DAMP mustard to inactivate M_3 receptors during a *Treatment Phase*, and subsequently, the contractile activity of muscarinic agonists is characterized during a *Test Phase* in the presence of histamine and a relaxant agent. When present together, histamine and the relaxant agent (e.g., isoproterenol or forskolin) have no net contractile effect because their actions oppose one another. However, under these conditions, muscarinic agonists elicit a highly potent contractile response through the M_2 receptor, presumably by inhibiting the relaxant action of isoproterenol or forskolin on histamine-induced contractions. This contractile response is pertussis toxin-sensitive, unlike the standard contractile response to muscarinic agonists, which is pertussis toxin-insensitive. When measured under standard conditions (i.e., in the absence of histamine and without 4-DAMP mustard-treatment), the contractile response to muscarinic agonists is moderately sensitive to pertussis toxin if isoproterenol or forskolin is present. Also, pertussis toxin-treatment enhances the relaxant action of isoproterenol in the field-stimulated guinea pig ileum. These results demonstrate that endogenous acetylcholine can activate M_2 receptors to inhibit the relaxant effects of ß-adrenoceptor activation on M_3 receptor-mediated contractions. An operational model for the interaction between M_2 and M_3 receptors shows that competitive antagonism of the interactive response resembles an M_3 profile under most conditions, making it difficult to detect the contribution of the M_2 receptor.

Key Words: muscarinic receptors, smooth muscle, gastrointestinal tract, muscarinic receptor-mediated contraction

Muscarinic receptors are widely distributed in smooth muscle throughout the body including that of the gastronintestinal tract (for reviews see (1-4)). The principle subtypes on the sarcolemma are the M_2 and M_3, with the M_2 receptor being expressed in about three- to four-fold greater abundance. When isolated strips of gastrointestinal smooth muscle are exposed to muscarinic agonists, the resulting contractions are sensitive to competitive antagonists in a manner consistent with an M_3 mechanism (5-10). This behavior can be explained by the coupling of M_3 receptors to the G_q family of G proteins, resulting in activation of phospholipase C-ß, phosphoinositide

hydrolysis and Ca^{2+} mobilization (11). The apparent lack of involvement of the M_2 receptor under these conditions can be explained by the coupling of this receptor to the G_i family of G proteins (11,12). For the most part, this pathway does not directly cause Ca^{2+} mobilization, but it can affect contraction under certain conditions. During the past few years, our laboratory has uncovered some conditions under which the M_2 receptor contributes to the contractile response in smooth muscle of the gastrointestinal tract and trachea. Our primary goal has been to discover new muscarinic responses; consequently, we have taken a pharmacological approach since this strategy is one of the best ways to assess receptor function. Our results also shed some light on contractile mechanisms. Here, we describe a summary of our progress.

Special Conditions are Required to Demonstrate Contractions through the M_2 Receptor

One of the clearest ways to determine the function of a receptor is to study the receptor in isolation. Such strategy has enjoyed great success in determining the signaling pathways of recombinant receptors in transfected cells. However, in smooth muscle, M_2 receptors are coexpressed with M_3 receptors, making it difficult to assess the contractile function of one without interference from the other. Moreover, M_2 selective agonists are unavailable. Consequently, we developed a strategy for inactivating non-M_2 muscarinic receptor subtypes selectively using the irreversible muscarinic antagonist, 4-DAMP mustard (13-15).

The impetus for our initial experiments came from the observation that M_2 receptors mediate an inhibition of adenylyl cyclase in transfected cells (11,16) and native smooth muscle (10,17,18). Since cAMP has a relaxant effect on smooth muscle, Candell and coworkers (10,19) proposed that M_2 receptors mediate an inhibition of the relaxant action of cAMP stimulating agents, like isoproterenol, without causing a direct contractile effect in their absence. To detect such a function, we designed the following experiment, which is divided into two epochs (9). In the first phase (*Treatment Phase*), isolated smooth muscle is treated with 4-DAMP mustard in combination with AF-DX 116 ([[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3b][1,4]-benzodiazepine-6-one) to inactivate M_3 receptors selectively. This treatment is followed by extensive washing. In the second phase (*Test Phase*), the smooth muscle is exposed to a non-muscarinic contractile agent, like histamine, followed by a cAMP-stimulating relaxant agent, like isoproterenol. The contractile effects of a muscarinic agonist are measured in the continued presence of histamine and isoproterenol during the *Test Phase*. In combination, histamine and isoproterenol have no net contractile effect because their actions oppose one another. However, if the M_2 receptor mediates an inhibition of the relaxant effect of isoproterenol on histamine-induced contractions, then muscarinic agonists should elicit contraction through the M_2 receptor during the *Test Phase*. If so, the contractile response would satisfy the requirements for using competitive antagonism to identify the muscarinic receptor mediating contraction during the *Test Phase*. Since the purpose of the complicated experimental paradigm is, in part, to satisfy these requirements, it is relevant to enumerate them: 1) the agonist must act on a single receptor subtype to elicit the response; 2) in the absence of the muscarinic agonist, there is no response; and 3) the response must be a continuous, increasing function of the agonist concentration. Factors that influence the occupancy-response relationship (i.e., histamine and isoproterenol) do not invalidate the analysis of competitive antagonism. Therefore, it should be possible to measure the dissociation constants of muscarinic antagonists (K_B vales) for the M_2 receptor using standard methods of competitive antagonism during the *Test Phase*.

In our initial experiments, we found that M_2 receptors mediate an inhibition of the relaxant effects of both isoproterenol and forskolin on histamine-induced contractions in the isolated guinea pig ileum (9). That is, the K_B values of muscarinic antagonists measured in the *Test phase*, were in excellent agreement with the binding affinities (K_D values) of the same antagonists for the cloned human M_2 receptor expressed in Chinese hamster ovary cells (3,20). Equally important, these K_B values did not agree with the corresponding K_D values for the cloned M_1, M_3, M_4 and M_5 subtypes (3). Moreover, the contractile response of the *Test Phase* was pertussis toxin-sensitive, unlike the standard guinea pig ileum bioassay which is pertussis toxin-insensitive (21). Finally, the potency of oxotremorine-M for eliciting contractions during the *Test Phase* in the guinea pig ileum often exceeded that measured in the standard guinea pig ileum bioassay (22). This high sensitivity in the face of excessive M_3 receptor inactivation attests to the significance of the M_2 response.

TABLE I

Interactions among M_2 Receptors and other Relaxant (*Category 1*) and Contractile (*Category 2*) Agents

Smooth muscle type (species)	*Category 1 agent (receptor)*	*Category 2 agent (receptor)*	*References*
Ileal, longitudinal (guinea pig)	Isoproterenol ($ß_1$, $ß_3$)	Histamine (H_1)	Thomas et al. (9,21); Reddy et al. (23)
		Muscarinic agonists; (M_3)	Thomas et al. (21); Ostrom and Ehlert (24)
	Forskolin (Adenylyl cyclase)	Histamine (H_1)	Thomas et al. (9,21)
		Muscarinic agonists; (M_3)	Ostrom and Ehlert (24)
	Dopamine (?)	Histamine (H_1)	Ostrom and Ehlert (22)
		Muscarinic agonists; (M_3)	Ostrom and Ehlert (22)
Colonic, longitudinal (guinea pig)	Forskolin (Adenylyl cyclase)	Histamine (H_1)	Sawyer and Ehlert (25)
	Isoproterenol (ß)	Histamine (H_1)	Sawyer and Ehlert (25)
Esophageal (rat)	Serotonin; ($5\text{-}HT_4$)	U46619 (TP)	Eglen et al. (26)
	Isoproterenol ($ß_3$)	U46619 (TP)	Eglen et al. (26)
Urinary bladder (rat)	Isoproterenol (ß)	KCl	Hedge et al. (27)
Tracheal [a] (cow)	Forskolin; (Adenylyl cyclase)	Histamine (H_1)	Ostrom and Ehlert (28)
		Muscarinic agonists; (M_3)	Ostrom and Ehlert (28)
Tracheal [b] (guinea pig)	Forskolin; (Adenylyl cyclase)	Histamine (H_1)	Thomas and Ehlert (29); Ostrom and Ehlert (24)
		Muscarinic agonists; (M_3)	Ostrom and Ehlert (24)
Tracheal (dog)	Isoproterenol ($ß_2$)	Acetylcholine (M_3)	Mitchell et al. (30)

[a] M_2 receptors do not mediate an inhibition of isoproterenol-mediated relaxation in bovine trachea (Roffel et al. (31); Ostrom and Ehlert (28))

[b] M_2 receptors do not mediate an inhibition of isoproterenol-mediated relaxation in guinea pig trachea (Roffel et al. (32); Watson et al. (33); Ostrom and Ehlert (24)).

The indirect role for the M_2 receptor described in the preceding paragraph raises questions about the nature of the receptors that interact with the M_2 receptor. These receptors can be divided into two categories: category 1, those mediating relaxation that is opposed by the M_2 receptor, and category 2, those mediating contraction that is opposed by the receptors of category 1. Table I summarizes results from several studies in which the experimental paradigm described above was used to determine the relaxant (category 1) and contractile (category 2) receptors that interact with the M_2 receptor in different tissues. We have also included results from some experiments using a different approach to determine whether the M_2 receptor inhibits relaxant effects on M_3 receptor-mediated contractions. These experiments are described in greater detail in the next section. Inspection of Table I shows that the M_2 receptor opposes the relaxant action of isoproterenol and forskolin on the contractile response elicited by H_1 histamine and M_3 muscarinic receptors in various smooth muscles of the gastrointestinal tract. Interestingly, M_2 receptors inhibit the relaxant effects of forskolin on H_1 histamine- and M_3 receptor-mediated contractions in the bovine and guinea pig trachea, but not the relaxant effects of isoproterenol (28,31-33), even though M_2 receptors mediate an inhibition of isoproterenol-stimulated cAMP accumulation in trachea (17,28,34,35). These results are consistent with the hypothesis of Torphy (36), that ß-adrenoceptors mediate relaxation through non-cAMP-dependent mechanisms in the trachea, perhaps through activation of Ca^{2+}-activated potassium channels (37,38). In canine trachea, M_2 receptors inhibit the relaxant effect of isoproterenol on M_3-receptor mediated contractions (30), and M_2 receptors have been shown to inhibit isoproterenol-induced activation of Ca^{2+}-activated potassium channels in canine trachea (39).

M_2 and M_3 Receptors Interact to Mediate Contraction in the Presence of Relaxant Agents

Pharmacological experiments on isolated tissues may reveal what function a receptor can have, yet they do not prove that the receptor participates in this function in the intact animal. This concern could be raised about the M_2 contractile mechanism described above. To address this issue, we considered two questions. First, does the M_2 response show dominance in the absence of 4-DAMP mustard-treatment when both M_2 and M_3 receptors are activated? Second, can endogenous acetylcholine elicit contraction through the M_2 receptor? To address the first question, we measured the effects of pertussis toxin on the contractile response to a muscarinic agonist in the presence of isoproterenol or forskolin. As mentioned above, pertussis toxin does not inhibit the contractile response to muscarinic agonists in the standard guinea pig ileum bioassay, which is mediated through the M_3 receptor. Also, in smooth muscle, pertussis toxin does not inhibit M_3 receptor-mediated phosphoinositide hydrolysis, but it does prevent M_2 receptor-mediated inhibition of adenylyl cyclase. We found that the contractile response to oxotremorine-M in the guinea pig ileum was moderately pertussis toxin-sensitive when measured in the presence of isoproterenol or forskolin (21,24). These results show that, in the absence of 4-DAMP mustard treatment, the M_2 receptor contributes to the contractile response when cAMP-stimulating relaxant agents are present. Presumably, M_2 receptors mediate an inhibition of relaxant effects on M_3-mediated contractions. Table I includes a summary of those tissues in which this interaction occurs. If M_2 and M_3 receptors interact to elicit contraction in the presence of cAMP-stimulating, relaxant agents, then one might expect the pharmacological antagonism of the interactive response to exhibit properties midway between M_2-like and M_3-like. However, we found the antagonism of muscarinic responses in the presence of isoproterenol to be almost entirely M_3-like in the guinea pig ileum (9). As shown in the next section, these results can be attributed to the nature of the interaction between M_2 and M_3 receptors. This interaction can be readily detected with pertussis toxin, but not the currently available M_2 selective, muscarinic antagonists.

To determine whether endogenous acetylcholine can elicit contraction through the M_2 receptor, we carried out experiments on the field-stimulated guinea pig ileum. The twitch response of this preparation is pertussis toxin-insensitive (40-42), and it is mediated almost entirely through the action of acetylcholine on M_3 muscarinic receptors (43-46). Nevertheless, we have found that the relaxant effects of forskolin and isoproterenol on the field stimulated guinea pig ileum are greatly potentiated by pertussis toxin-treatment (Sawyer and Ehlert, unpublished observations). Pertussis toxin is without effect on the ability of isoproterenol and forskolin to inhibit H_1 mediated contractions. Thus, our results show that endogenous acetylcholine can act on M_2 receptors to inhibit the relaxant effects of isoproterenol and forskolin on the contraction mediated by endogenous acetylcholine acting through M_3 receptors. Such a mechanism may play a role in the gastrointestinal tract when parasympathetic nerves oppose the action of sympathetic nerves.

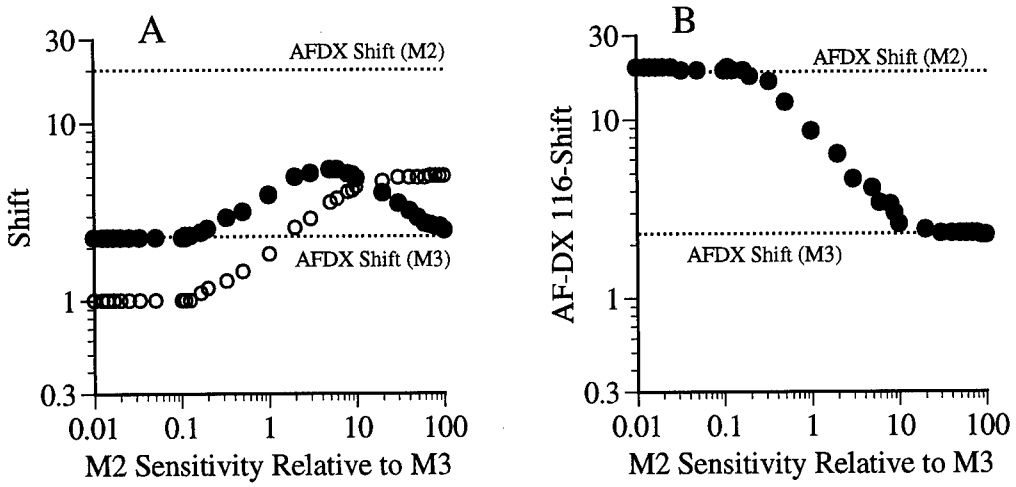

Fig. 1

Models for the interaction between M_2 and M_3 receptors. The solid lines indicate signaling pathways that are capable of eliciting contraction, whereas the dashed lines indicate pathways that are silent by themselves, but are capable of amplifying other signals to trigger a contractile response.

The Competitive Antagonism of Responses Mediated by Interacting Receptors is Complex

Since the action of muscarinic agonists on smooth muscle can be attributed to a stimulation of both M_2 and M_3 receptors when cAMP-stimulating agents are present, we wondered about the ability of subtype selective muscarinic antagonists to inhibit such an interaction. Consequently, we developed two models for a response mediated through the interaction of M_2 and M_3 receptors (see Figure 1) and used an operational model to simulate the competitive inhibition of the interactive response. In Model I, the M_3 receptor mediates a direct contractile response. Activation of the M_2 receptor has no effect by itself, but this activation tunes up the response elicited by the M_3 receptor. In model II, both the M_2 receptor and the M_3 receptor are inactive by themselves, but when stimulated simultaneously, their combined effects trigger a productive response. Model II is equivalent to an AND gate in electronics. To predict the behavior of these two models, we used a strategy based on the operational model of Black and Leff (47). In our analysis, we assumed that the muscarinic agonist has the same affinity for M_2 and M_3 receptors, and that the response to the agonist is a function (f) of the stimulus as defined by Stephanson (48). We also assumed that stimulus-response function (f) obeyed a logistic equation described by a sensitivity constant (β) and a cooperativity exponent (n) with $n = 2$ (see Black and Leff (47)). The details of our model are described elsewhere (Sawyer and Ehlert, submitted). Briefly, Model I is based on the assumption that the response or amplifying signal of the M_2 receptor increases the stimulus generated by the M_3 receptor. The maximal response of the M_2 receptor is to cause a five-fold increase in the M_3 stimulus. This amplified M_3 stimulus is then submitted to the M_3 stimulus-response function to simulate the response at various agonist concentrations. For Model II, the M_3 stimulus is multiplied by the M_2 response which increases from zero to one with increasing agonist concentration. The resulting M_3 stimulus is then submitted to the M_3 stimulus response function to calculate response. Using this model, the effects of selective muscarinic antagonists can be simulated. Also, the effects of pertussis toxin can be simulated by eliminating the contribution of the M_2 receptor.

Figure 2A shows the results of our simulations for Model I. The shift in the concentration-response curve caused by the M_2 selective antagonist, AF-DX 116, at a concentration of 1 μM is plotted with the closed symbols (●) as a function of the relative sensitivity of the M_2 signal. In the operational model, sensitivity is proportional to the reciprocal of the parameter β (see above paragraph), with high sensitivity corresponding to a low β value. The theoretical AF-DX 116-induced shift for purely M_2-mediated and M_3-mediated responses are shown by the upper and lower dotted lines, respectively. When the sensitivity of the M_2 amplifying signal is much less than

that of the M_3 response ($\beta_2 >> \beta_3$), then there is no interaction, and the M_3 receptor is able to elicit a maximal contraction at agonist concentrations that are too low to elicit an M_2 signal. Consequently, the antagonism resembles an M_3-mediated response under these conditions. Similarly, when the sensitivity of the M_2 signal is much greater than that of the M_3 receptor ($\beta_2 << \beta_3$), the antagonism also resembles an M_3 mediated response. This behavior occurs because the relationship between the agonist concentration and the M_2 signal can be greatly shifted to the right by AF-DX 116, yet

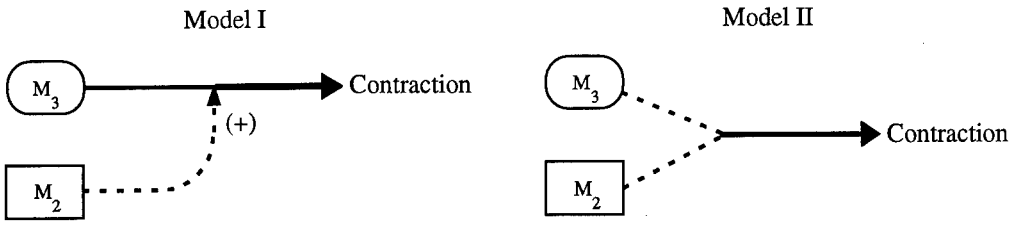

Fig. 2

Simulation of the effects of AF-DX 116 (●) and pertussis toxin (○) on a response mediated by Model I (A) and Model II (B). The dotted lines indicate the predicted competitive shifts caused by AF-DX 116 (1.0 μM) at an M_2-mediated response (AFDX shift (M2)) and an M_3-mediated response (AFDX shift (M3)).

nevertheless, it can still be more sensitive than the relatively insensitive M_3 response. Therefore, AF-DX 116 would not cause a diminution in M_2 amplification under these conditions. It is only when the sensitivity of the M_2 signal is about five-fold greater than the M_3 response that the antagonistic effects of AF-DX 116 become prominent (five- to six-fold shift). Even under these conditions, the AF-DX 116-induced shift is much less than that expected for antagonism of an M_2 response (20-fold shift). The effects of pertussis toxin on Model I are shown by the open symbols (○). It can be seen that, as the sensitivity the M_2 receptor exceeds that of the M_3 receptor, the interactive response becomes pertussis toxin-sensitive. Thus, when the sensitivity of the M_2 signal is much greater than that of the M_3 response, Model I predicts that the interactive response should exhibit an M_3 antagonistic profile, yet be pertussis toxin-sensitive. This model explains why the contractile response to oxotremorine-M in the guinea pig ileum is pertussis toxin-sensitive in the presence of isoproterenol, yet nevertheless, it is only weakly antagonized by AF-DX 116. We have previously described this rationale on a less mathematical basis (3,21).

Figure 2B shows the results of our simulations for Model II. For this interaction, where the response depends on the activation of both receptor subtypes, the rules are simple - the pharmacological antagonism resembles that expected for the less sensitive receptor signaling mechanism. Since the response always depends on activation of the M_2 receptor as well as the M_3, it is completely pertussis toxin-sensitive. Thus, when the sensitivity of the M_2 signal is much greater than that of the M_3 response, Model II predicts that the interactive response should be pertussis toxin-sensitive, yet exhibit an M_3 antagonistic profile. This situation could arise after the sensitivity of the M_3 response has been greatly inhibited by 4-DAMP mustard treatment. An example of this situation is described next.

M_2 and M_3 Receptors Interact to Mediate Contraction in the Absence of Relaxant Agents

So far, we have only described a role for the M_2 receptor in smooth muscle when there is active relaxation mediated through cAMP. Recently, we have observed an unusual interaction between M_2 and M_3 receptors in the guinea pig colon that is readily demonstrable in the absence of any relaxant agents (25). In the guinea pig colon, the contractile response to muscarinic agonists is pertussis toxin-insensitive, like that of many other smooth muscles. However, following extensive M_3 receptor inactivation, the residual contractile response is pertussis toxin-sensitive. This

sensitivity suggests a role for the M_2 receptor in contraction. Interestingly, the antagonistic profile of this residual contractile response is M_3-like. Thus, this response resembles the enigmatic behavior of Models I and II which show that an interaction between M_2 and M_3 receptors can result in a pertussis toxin-sensitive response with an M_3 antagonistic profile. Although the mechanism for this interaction is unclear, it bears a resemblance to the muscarinic induced nonselective cation conductance, which is a pertussis toxin-sensitive, M_2 gated conductance that depends on M_3 receptor activation (49-52). The physiological significance of this contractile response in the colon is questionable because it is only observed after extensive M_3 receptor inactivation. This requirement may represent a limitation in the isolated tissue bath experiment where a global activation of muscarinic receptors occurs following application of an agonist. It is possible that Model II is the dominant mechanism at a subset of the cholinergic neuroeffector junctions in the colon, whereas the standard M_3 receptor-mediated contraction may exhibit a different postjunctional display.

Acknowledgments

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MUSCARINIC ANTAGONISTS IN DEVELOPMENT FOR DISORDERS OF SMOOTH MUSCLE FUNCTION

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Summary

Compounds with high affinity for muscarinic M_3 receptors have been used for many years to treat conditions associated with altered smooth muscle tone or contractility such as urinary urge incontinence, irritable bowel syndrome or chronic obstructive airways disease. M_3 selective antagonists have the potential for improved toleration when compared with non-selective compounds. Darifenacin has high affinity (pKi 9.12) and selectivity (9 to 74-fold) for the human cloned muscarinic M_3 receptor. Consistent with this profile, the compound potently inhibited M_3 receptor mediated responses of smooth muscle preparations (guinea pig ileum, trachea and bladder, pA_2 8.66 to 9.4) with selectivity over responses mediated through the M_1 (pA_2 7.9) and M_2 receptors (pA_2 7.48). Interestingly, darifenacin also exhibited functional tissue selectivity for intestinal smooth muscle over the salivary gland. The M_3 over M_1 and M_2 selectivity of darifenacin was confirmed in a range of animal models. In particular, in the conscious dog darifenacin inhibited intestinal motility at doses lower than those which inhibit gastric acid secretion (M_1 response), increase heart rate (M_2 response) or inhibit salivary secretion. Clinical studies are ongoing to determine if darifenacin has improved efficacy and or toleration when compared with non-selective agents.

Key Words: muscarinic antagonists, smooth muscle, darifenacin

Five subtypes of human muscarinic receptors have been identified and cloned and these have been designated M_1, M_2, M_3,M_4 and M_5. Pharmacologically, M_1, M_2, M_3 and M_4 receptor subtypes correlate with the M_1, M_2, M_3 and M_4 gene products (1), although a pharmacological correlate of the M_5 gene is only just being defined. Both M_2 and M_3 receptors are expressed in smooth muscle and the M_2 receptor accounts for approximately 80% of the total receptor population (2). Although the M_2 receptor expression is very high in smooth muscle its function has yet to be fully defined since under normal physiological conditions the functional response of smooth muscle is mediated through the M_3 receptor. Agents with high affinity for muscarinic M_3 receptors have utility in the treatment of a range of disorders associated with altered smooth muscle contractility and tone such as, urinary incontinence, irritable bowel syndrome and chronic obstructive airways disease. The clinical utility of available muscarinic receptor antagonists is limited by the lack of selectivity leading to the classical antimuscarinic side effects such as dry mouth, blurred vision, tachycardia and effects on cognitive function. Selective M_3

antagonists have the potential to inhibit smooth muscle contractility in the absence of M_1 and M_2 mediated side effects. A research programme directed at identify M_3 receptor antagonists has culminated in the identification of ((S)-2-{1-[2-(2,3,-dihydrobenzfuran-5yl)ethyl]-3-pyrrolidinyl}-2,2-diphenylacetamide) (Figure 1) (3)

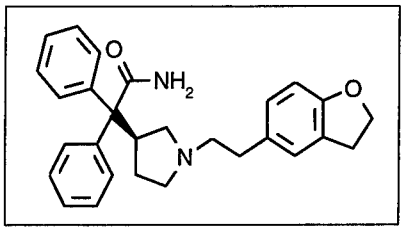

Figure 1
Structural Formula of Darifenacin

In vitro pharmacology

The binding affinities of darifenacin in comparison to other muscarinic antagonists with high affinity for the M_3 receptor has been determined using membranes from CHO-K1 cells stably expressing human M_1 - M_5 receptors. The experiments were conducted at 25°C using 20 mM HEPES buffer using [^{3}H]-NMS as the radioligand. Under these conditions darifenacin had high affinity and selectivity for the M_3 receptor and is one of the few compounds which exhibited preferential affinity for one receptor subtype (Table I). In contrast, tolterodine, like atropine, was non-selective. These data are consistent with previous reports showing tolterodine does not differentiate between muscarinic receptor subtypes (4). Like tolterodine, dicyclomine and oxybutynin did not differentiate between muscarinic M_1 and M_3 receptors, although some selectivity over M_2, M_4 and M_5 receptors was observed.

TABLE I

Binding Profile of Muscarinic Antagonists to the Human Cloned Muscarinic Receptors

	M_1	M_2	M_3	M_4	M_5
Darifenacin	8.15 ± 0.04	7.35 ± 0.11	9.12 ± 0.08	7.34 ± 0.08	8.03 ± 0.08
Tolterodine	8.75 ± 0.01	7.98 ± 0.07	8.53 ± 0.07	7.67 ± 0.07	7.74 ± 0.03
Oxybutynin	8.69 ± 0.04	7.77 ± 0.05	8.86 ± 0.06	8.02 ± 0.04	7.43 ± 0.03
Dicyclomine	8.70 ± 0.03	6.89 ± 0.08	8.32 ± 0.04	7.54 ± 0.08	7.78 ± 0.05
Atropine	9.55 ± 0.03	8.88 ± 0.07	9.56 ± 0.10	8.94 ± 0.03	9.18 ± 0.03

Results are mean ± S.E.M. pKi values (n= 4-8), Hill slopes did not differ from unity.

Recently darifenacin has been radiolabelled and has proved to be a valuable ligand. [^{3}H]-Darifenacin binds with high affinity to the M_3 receptor (K_D 0.33 nM) and with selectivity over the M_1 receptor (K_D 1.6 nM); no specific binding to M_2, M_4 and M_5 receptors was detected (5). This radioligand should prove to be a valuable tool to study the tissue distribution and pharmacology of M_3 receptors. Preliminary data from experiments comparing the binding of [^{3}H]-darifenacin with [^{3}H]-n-methylscopolamine ([^{3}H] NMS) (which binds non-selectively to M_1 - M_5) to homogenates of guinea pig brain indicate that as much as 80% of the sites bound by [^{3}H]-NMS are also bound by [^{3}H]-darifenacin. As Figure 2 shows, scatchard analysis of saturation curves for [^{3}H]-darifenacin are curvilinear consistent with binding to M_1 and M_3 receptors. Experiments are in progress to estimate the relative proportions of M_1 and M_3 bound by [^{3}H]-darifenacin. This contrasts the situation in the guinea pig bladder where similar experiments indicate [^{3}H]-darifenacin binds to one population of receptors representing 40-60% of total muscarinic receptors. This estimation of the M_3 receptor population in the bladder is slightly higher than previous estimates (2) and this may reflect differences in methodology since these studies are the first to use a M_3 selective ligand.

The profile of these muscarinic antagonists in functional studies was similar to that observed in the above radioligand binding studies, although the magnitude of M_3 selectivity was much greater (Table II). Thus, darifenacin was a potent antagonist in smooth muscle preparations such as the guinea pig ileum, bladder and trachea as well as on the human isolated bladder (pKb 9.72 ± 0.19) (6). These data suggest that the contractile responses of the human bladder is also mediated via the M_3 receptor. Interestingly, in studies investigating muscarinic receptors controlling salivary secretion, darifenacin was shown to be 6-fold weaker than atropine as an inhibitor of carbachol-stimulated ^{86}Rb efflux from the guinea pig submandibular gland despite having similar potency on the ileum (7). Other compounds, such as zamifenacin, p-F-HHSiD and RDS-127, have also demonstrated functional tissue selectivity (8). Indeed tolterodine has shown some functional bladder selectivity over the salivary gland (4). The molecular basis for this tissue selectivity remains unknown, but [^{3}H]-darifenacin may prove to be a valuable tool to investigate its basis. Similar results have been demonstrated using other receptor systems. In functional studies the cloned α_{1A} receptor displays pharmacological properties consistent with those of the putative α_{1L} receptor found in the prostate and this is distinct from the profile obtained in membrane binding studies using the same cell line (9). Therefore, in a single cell line the pharmacology of a gene product can vary depending on the technique (binding compared with functional activity) used to investigate the properties of that receptor. Again the molecular basis for this observation is unknown, but these results emphasis the need for a combination of techniques to fully characterise new chemical entities and to classify receptor subtypes. Alternatively, functional responses in tissues may be controlled by multiple receptors. For example, salivation in response to pilocarpine is reduced in the M_5 knockout mouse (Yeomans *et al*, this meeting) suggesting a role for M_5 receptors in the control of salivary secretion. Thus the M_3 over M_5 selectivity of darifenacin may contribute to its smooth muscle over salivary gland selectivity.

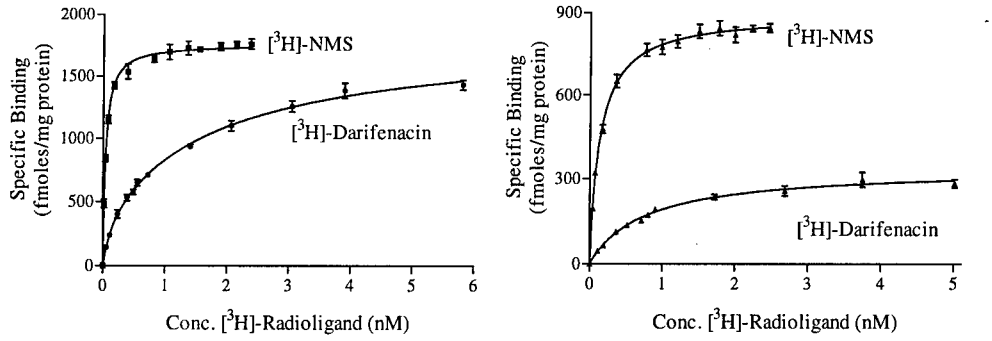

Figure 2
Saturation analysis for guinea pig brain (A) and bladder (B).

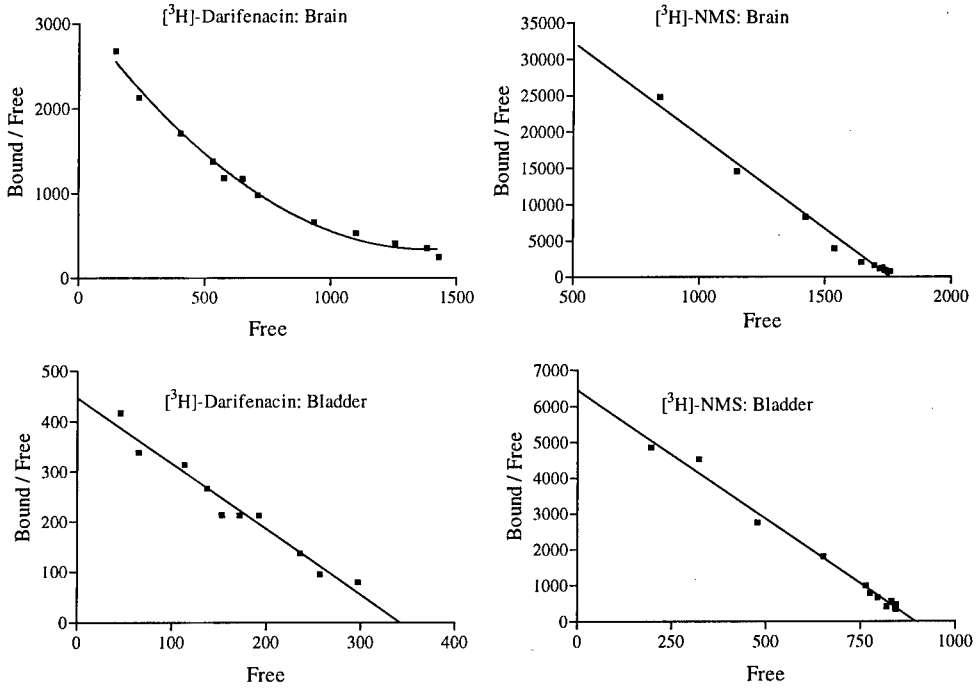

Figure 3
Scatchard Analysis of data shown in Figure 2.

TABLE II

Functional Profiles of Muscarinic Antagonists.

	Guinea Pig Ileum	Guinea Pig Bladder	Guinea Pig Trachea	Guinea Pig Atria	Rabbit Vas Deferens
Darifenacin	9.44 ± 0.07	8.66 ± 0.14	8.70 ± 0.09	7.48 ± 0.13	7.90 ± 0.08
Atropine	9.40 ± 0.07	9.01 ± 0.09	9.20 ± 0.10	8.72 ±0.06	9.54 ± 0.09
Dicyclomine	7.75 ± 0.09	-	-	6.09 ± 0.17	7.70 ± 0.08[1]
Tolterodine[2]	-	7.77 ± 0.06	-	7.68 ± 0.25	8.49 ± 0.17
Oxybutynin	-	7.44 ± 0.16	-	7.12 ± 0.29	7.90 ± 0.14

Results are mean ± S.E.M. pA_2 values, n= 5-8, [1] data taken from (10), [2] data taken from (7)

In vivo pharmacology

In anaesthetised dogs, small bowel motility was stimulated by intravenous administration of the hormone, cholecystokinin. In the same animals salivary secretion was induced by stimulation of the duct of the submandibular gland. Intravenous infusions of darifenacin potently inhibited stimulated small bowel motility (ED_{50} 0.32 mcg/kg/min) in the absence of effects on heart rate (11). Significantly, in the same experiments, darifenacin was less potent as an inhibitor of salivary secretion (ED_{50} 2.16 mcg/kg/min) induced by electrical stimulation of the duct of the submandibular gland. In contrast, atropine inhibited small bowel motility over the same dose range as that which inhibited salivary secretion and increased heart rate (ED_{50} 0.26 to 0.36 mcg/kg/min). Oral activity of darifenacin has been demonstrated in conscious dogs instrumented to record heart rate and jejunal motility. The wetness of the mouth was measured via pre-weighed swabs placed in the cheek jowls as a measure of salivary secretion. Darifenacin inhibited small bowel digestive motility in a dose-related manner (ED_{50} 0.1 mg/kg p.o.) in the absence of effects on heart rate and with reduced effects on salivary secretion (ED_{50} 0.3 mg/kg p.o.) (Table III, (12)).

A similar smooth muscle selectivity was observed in the conscious rat (13). In these studies animals were surgically prepared to record bladder cystometry in response to saline infusions into the bladder or salivary secretion in response to subcutaneous methacholine. Darifenacin caused a dose-related inhibition of micturition pressure (ED_{50} 0.089 mg/kg i.v.) with concomitant changes in micturition interval and volume, at doses lower than those required to inhibit salivary secretion (ED_{50} 0.48 mg/kg i.v.). Thus, although the molecular basis for the functional smooth muscle selectivity of compounds, such as darifenacin, is unknown it does translate from *in vitro* through to *in vivo* studies. The fact that the effects on smooth muscle contractility and salivary secretion were measured in the same animals at the same time would argue against experimental artefacts complicating the interpretation of the data.

In addition to inhibiting intestinal motility darifenacin has also been shown to increase intestinal compliance (14). Thus following darifenacin, the pressure in the bowel at a given volume will

be less. This may provide an additional benefit in the treatment of conditions associated with altered gut motility patterns.

Muscarinic M_1 receptors are involved in the control of gastric acid secretion (15) and cognitive function (16) and therefore inhibition of gastric acid secretion can be used as a measure of muscarinic M_1 receptor blockade. Gastric acid secretion was stimulated by intravenous infusions of pentagastrin in Heidenhain pounch dogs. Pirenzepine (ED_{50} 0.037 mg/kg p.o.), but not methroctramine (ED_{50} >1.0 mg/kg p.o.), potently inhibited gastric acid secretion confirming the role of M_1 receptors in this model (17). Atropine also inhibited acid secretion following doses lower than those active on small bowel motility (Table III). In contrast, darifenacin had no significant effect on acid secretion following doses as high as 3.0 mg/kg p.o.. These data are consistent with the functional M_3 over M_1 selectivity (30-fold) of darifenacin and demonstrate that compounds which are selective over muscarinic M_1 receptors will have less effect on gastric acid secretion. The ability to confirm the M_3 selectivity of darifenacin *in vivo* would suggest that the compound may have less adverse effects on cognitive function than non-selective agents.

TABLE III

Effects of Darifenacin and Atropine on Heart Rate, Small Bowel Digestive Motility and Gastric Acid in the Conscious Dog (n= 4-6).

	Small Bowel motility (ED_{50})	Salivary Secretion (ED_{50})	Gastric Acid Secretion (ED_{50})	Heart rate (ED_{150})
Darifenacin	0.1	0.3	>3.0	>3.0
Atropine	0.04	0.06	0.007	0.2

The above preclinical studies have shown that darifenacin has high affinity for M_3 receptors over the other muscarinic receptor subtypes. This results in a preferential effect on smooth muscle function in the absence of effects on heart rate (M_2) or gastric acid secretion (M_1). These effects are consistent with the known pharmacology of muscarinic receptors

Clinical Pharmacology

Tolterodine has been recently approved by the FDA for the treatment of the over active bladder. The drug has been shown to reduce the frequency of micturition and the number of incontinent episodes (17). Efficacy was achieved with a reduced incidence of moderate/severe episodes of dry mouth. These data would suggest that the functional tissue selectivity of muscarinic antagonists can translate to man. A low dose (1 mg) of tolterodine was associated with a higher incidence of palpitations than placebo, although this was not seen with a higher dose (2 mg) suggesting these effects may not be drug related (18). Alternatively an increased incidence of tachycardia may reflect the lack of M_3 over M_2 selectivity of tolterodine. Data from the Phase II trials with darifenacin has yet to be published and so it remains to be proven whether the M_3 selectivity of this compound translates to improved toleration in man.

Conclusions

Compounds with high affinity for muscarinic M_3 receptors have clinical utility in the treatment of conditions associated with altered smooth muscle contractility and or tone. Darifenacin has been shown to be selective for the M_3 receptor over other muscarinic receptor subtypes both *in vitro* and *in vivo* and to exhibit some functional tissue selectivity for smooth muscle over the salivary gland. The recent approval of tolterodine for the treatment of the over active bladder has confirmed the clinical utility of compounds with high affinity for the M_3 receptor in the treatment of these conditions. Comparative clinical trials between M_3 selective agents such as darifenacin and non-selective compounds, such as, tolterodine are required to confirm the potential for improved toleration of receptor selective antagonists.

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NEURONAL SOMA-DENDRITIC AND PREJUNCTIONAL M_1 - M_4 RECEPTORS IN GASTROINTESTINAL AND GENITOURINARY SMOOTH MUSCLE

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Summary

A variety of neurons in gastrointestinal and genitourinary smooth muscle express muscarinic auto- as well as heteroreceptors. These receptors are found on the soma and dendrites of many cholinergic, sympathetic and NANC neurons and on axon terminals. A given neuron may contain both excitatory and inhibitory presynaptic muscarinic receptors. The subtypes involved are species- and tissue-dependent, and neuronal M_1 to M_4 receptors have been shown to be expressed in smooth muscle tissues. In this study, the ability of several selective muscarinic receptor antagonists to inhibit the effect of arecaidine propargyl ester (APE) on prejunctional muscarinic receptors on sympathetic nerve endings in the rabbit anococcygeus muscle (RAM) was investigated to characterise the receptor subtype involved. Electrical field stimulation (EFS) resulted in a release of noradrenaline (NA) eliciting monophasic contractions due to stimulation of postjunctional α_1-adrenoceptors. The selective muscarinic agonist APE did not reduce contractions to exogenous NA, but caused a concentration-related and N-methylatropine-sensitive inhibition of neurogenic responses. All muscarinic antagonists investigated failed to affect the EFS-induced contractions, but shifted the concentration-response curve of APE to the right in a parallel and surmountable fashion. Schild analysis yielded regression lines of unit slope, indicating competitive antagonism. The following rank order of antagonist potencies (pA_2 values) was found: tripitramine (9.10) > AQ-RA 741 (8.26) ≥ himbacine (8.04) ≥ (S)-dimethindene (7.69) > pirenzepine (6.46) ≥ p-F-HHSiD (6.27). A comparison of the pA_2 values determined in the present study with literature binding and functional affinities obtained at native or recombinant M_1 to M_5 receptors strongly suggests that NA release from sympathetic nerve endings in RAM is inhibited by activation of prejunctional muscarinic M_2 receptors.

Key Words: smooth muscle, presynaptic muscarinic receptors, rabbit anococcygeus muscle, noradrenaline release

Muscarinic receptors play an essential role in maintaining the contractile state of smooth muscle throughout the body. It is now evident that many smooth muscle cells coexpress M_2 and M_3 subtypes. The M_3 receptor mediates phosphoinositide hydrolysis and calcium mobilization, whereas the M_2 receptor mediates an inhibition of adenylyl cyclase. While the role of the post-

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junctional M_3 subtype in smooth muscle contraction is well established, recent data also implicate the M_2 receptor in the control of smooth muscle tone (1-4). A variety of neurons in smooth muscle express presynaptic muscarinic auto- and/or heteroreceptors, which serve to modulate the rate of neuronal firing and the release of neurotransmitters. These receptors are found on the soma and dendrites of many cholinergic, sympathetic and nonadrenergic-noncholinergic (NANC) neurons and on axon terminals. A given neuron may contain both excitatory and inhibitory presynaptic muscarinic receptors (5-9). The subtypes involved are species- and tissue-dependent.

Pharmacological and molecular cloning studies have revealed the existence of four neuronal muscarinic receptor subtypes, M_1 to M_4, in gastrointestinal and genitourinary smooth muscle. The *guinea-pig ileum* contains two types of neuronal muscarinic receptors. The inhibitory prejunctional autoreceptors have been classified as M_1 receptors in the circular muscle (10), and as M_3 in myenteric neurons of the longitudinal muscle (11). Muscarinic agonists cause a TTX-sensitive increase in spontaneous acetylcholine release from myenteric neurons (12) and a membrane depolarization (13) by stimulation of soma-dendritic M_1 autoreceptors. In the *rat perfused stomach*, stimulation-evoked release of endogenous acetylcholine was decreased by muscarinic receptor activation. Antagonist attenuation of this effect showed a profile in keeping with the involvement of an inhibitory prejunctional M_3 autoreceptor (14). The parasympathetic nerves innervating the *urinary bladder* are endowed with prejunctional facilitatory and inhibitory muscarinic receptors. The facilitatory autoreceptors have been classified as M_1 in the rat (15, 16) and rabbit urinary bladder (17). The inhibitory prejunctional muscarinic receptor appears to be M_2 or M_4 in the rat urinary bladder (16, 18), M_2 in the rabbit bladder (17) and M_4 in the guinea-pig bladder (19). In the *rat stomach*, it has been demonstrated that the oxotremorine-induced inhibition of stimulation-evoked release of endogenous noradrenaline (NA) from sympathetic nerve terminals is mediated by prejunctional muscarinic M_2 heteroreceptors (20). Stimulation of soma-dendritic muscarinic heteroreceptors, located on inhibitory nitrergic NANC neurons, causes a fall in lower *esophageal sphincter* pressure of the opossum (21, 22). These receptors are selectively activated by McN-A-343, and selectively antagonized by pirenzepine. Thus, Gilbert et al. designated the muscarinic receptors on the NANC neurons as M_1 (21). However, the data do not rule out the possibility that the M_4 receptor mediates the relaxant response in the esophageal sphincter. Muscarinic receptors mediating relaxation in *rat duodenum* via activation of inhibitory NANC neurons were reported to be of the M_1 or M_4 subtype (23, 24). Our recent demonstration of affinity estimates for a series of subtype-prefering muscarinic antagonists (25), e.g. pirenzepine (7.90), AQ-RA 741 (7.28) and himbacine (6.80), lends support to the original M_1 receptor classification of this soma-dendritic muscarinic heteroreceptor (23). Electrical field stimulation (EFS) of *rabbit vas deferens* results in a concomitant release of ATP and NA from sympathetic nerve endings eliciting a biphasic contractile response. Prejunctional muscarinic heteroreceptors have been described, which when activated cause inhibition of these effects (5, 26-28). The pharmacological profile (5, 26-28), transduction pathway (29) and immunoreactivity to specific anti-muscarinic receptor antibodies (30) clearly indicate that these prejunctional muscarinic receptors do not belong to the M_2, M_3 and M_5 subtypes. However, there are arguments for and against the presence of M_1 and M_4 receptors. Perhaps the data may be reconciled by multiple, M_1 and M_4, receptors or it may be that the prejunctional inhibitory muscarinic heteroreceptors in the vas deferens are the rabbit homologues of the mammalian M_1 or M_4 subtype.

In the *rabbit isolated anococcygeus muscle* (RAM) with histamine-raised tone activation of soma-dendritic muscarinic receptors has been reported to stimulate the release of nitric oxide from inhibitory NANC neurons, thereby causing relaxation (31). Antagonist attenuation of the relaxant effect of muscarine showed a profile in keeping with the involvement of a muscarinic M_4 receptor: AQ-RA 741 = himbacine > tripitramine ≥ p-F-HHSiD = methoctramine = pirenzepine > guanylpirenzepine (31). Thus, the isolated RAM is the first robust functional muscarinic M_4

receptor model. The RAM receives, in addition to an inhibitory NANC innervation, a dense adrenergic motor innervation via sympathetic nerves. In the absence of intrinsic tone, EFS results in a purely contractile response, mediated by the release of NA (32). The aim of the present study was to investigate whether the NA-induced neurogenic contractions in RAM are modulated by prejunctional muscarinic heteroreceptors.

Methods

Both anococcygeus muscles of male New Zealand white rabbits were incubated at 33°C in modified Krebs buffer, supplemented with L-tyrosine (0.01 mM) as NA precursor and with the NO-synthase inhibitor L-NOARG (0.3 mM) to suppress NO-induced NANC-relaxations (31). Unless stated otherwise, the Krebs buffer additionally contained cocaine (3 μM; inhibition of neuronal NA-uptake$_1$), corticosterone (3 μM; inhibition of extraneuronal NA-uptake$_2$) and yohimbine (30 nM; to block prejunctional inhibitory α_2-adrenoceptors). EFS (1 or 2 Hz; 0.5 ms; 35 - 40 V) was used to excite intramural nerves. In each experiment standard trains of 45 - 240 pulses were used at each stimulation frequency at intervals of 15 - 20 min, and neurogenic responses were recorded isotonically. The muscarinic agonist arecaidine propargyl ester (APE) was added to the bath fluid 4 min before a stimulation period and allowed to remain in contact until the neurogenic response had fully developed. The incubation time for other drugs was at least 30 min under continued electrical stimulation. To test effects of muscarinic antagonists concentration-response curves to APE were constructed in the absence and in the presence of 2 - 4 antagonist concentrations (log interval = 0.48).

Data analysis

The effects of drugs were expressed as a percentage in relative change of the neurogenic contraction compared to preceeding control responses (no drug present). The apparent potency of an agonist was expressed by its pEC_{50} value. Apparent affinities of the muscarinic antagonists (pA_2 values) were derived from Schild plots constrained to slope of unity. Results are given as arithmetic means ± S.E.M. from at least three independent experiments. Differences between mean values were tested for statistical significance by Student's *t* test; $P < 0.05$ was accepted as being significant.

Drugs used

Pirenzepine and AQ-RA 741 (Thomae, Biberach/Germany), himbacine (Dr. W.C. Taylor, Sydney/Australia), tripitramine (Dr. C. Melchiorre, Bologna/Italy), (S)-dimethindene (Zyma, Munich/Germany), p-fluoro-hexahydro-sila-difenidol (p-F-HHSiD; Dr. R. Tacke, Würzburg/Germany) and arecaidine propargyl ester (APE; Dr. U. Moser, Frankfurt/M./Germany). All other chemicals were of the highest grade available.

Results and Discussion

Sympathetic neurotransmission in RAM

In Krebs buffer not supplemented with yohimbine, cocaine and corticosterone, EFS at 2 Hz (40 V) produced tetrodotoxin (1 μM)-sensitive, rapid and reproducible contractions of the RAM, with a maximum response obtained within 45 to 240 s. Cocaine (0.3 - 10 μM) induced an increase in the neurogenic contractile response. This effect appeared to be maximal at a concentration of 2 μM (182 ± 12 %). Treatment of the preparations with corticosterone (1 - 10 μM) slightly enhanced EFS-induced contractions, but caused an up to 400 % increase of the

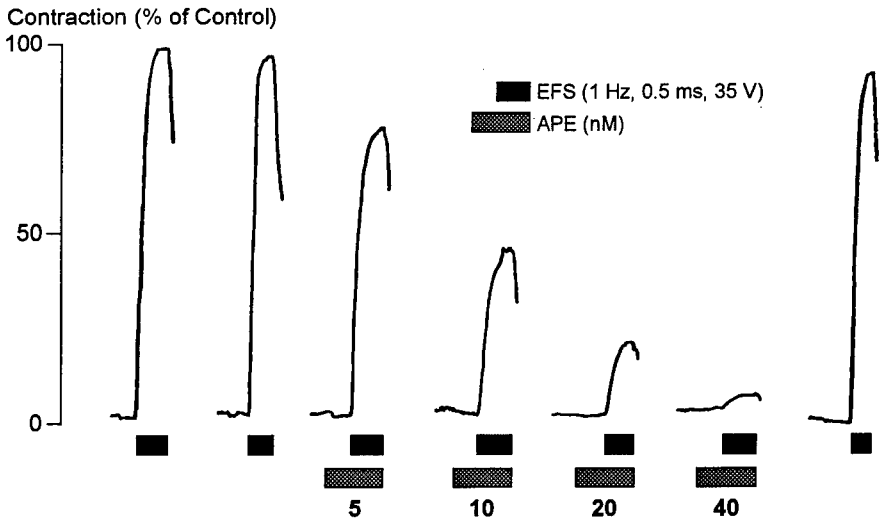

Fig. 1

Illustration of the effect of APE on response to EFS of the RAM. Trains of 45 - 240 pulses were used at intervals of 15 - 20 min. APE was incubated 4 min prior to the stimulation period and kept throughout.

neurogenic contractions in tissues pretreated with 5 μM cocaine (33). The α_2-adrenoceptor antagonist yohimbine (10 - 100 nM) increased contractile responses to EFS. This effect was maximal at 30 nM (189 ± 26 %). In order to minimize distortion of the EFS-induced contractions by elimination processes such as neuronal and extraneuronal NA uptake and by stimulation of inhibitory prejunctional α_2-adrenoceptors, all subsequent experiments were carried out in the continuous presence of cocaine (3 μM), corticosterone (3 μM) and yohimbine (30 nM). Since it is well known that the effectiveness of presynaptic inhibitory mechanisms is inversely related to the intensity of nerve stimulation (26), in all the following experiments trains of 45 - 240 pulses were applied at a stimulation frequency of 1 Hz (35 V).

At a stimulation frequency of 1 Hz the α_1-selective antagonist prazosin (0.3 - 30 nM; pIC_{50} = 8.54 ± 0.8) and guanethidine (1 - 10 μM; pIC_{50} = 5.56 ± 0.13) inhibited the neurogenic contractions to EFS in a concentration-dependent manner and abolished them at the highest concentration used. In contrast, the β-adrenoceptor antagonist propranolol (0.01 - 1 μM), the cyclooxygenase inhibitor indomethacin (0.1 - 10 μM), the P2 receptor antagonists suramin (100 μM) and pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (100 μM) (34) as well as the muscarinic receptor antagonist N-methylatropine (1 μM) did not significantly change the EFS-induced contractions in RAM.

These results confirm and extend the findings of Creed et al. (32) that EFS-induced contractions in RAM (in the presence of L-NOARG) are caused by exocytotic release of NA from sympathetic nerves acting at postjunctional α_1-adrenoceptors. Prejunctional facilitatory or postjunctional inhibitory β-adrenoceptors appear not to be involved. Under the present experimental conditions, the release of NA and the subsequent contractions are not modulated by endogenous acetylcholine, ATP or prostanoids.

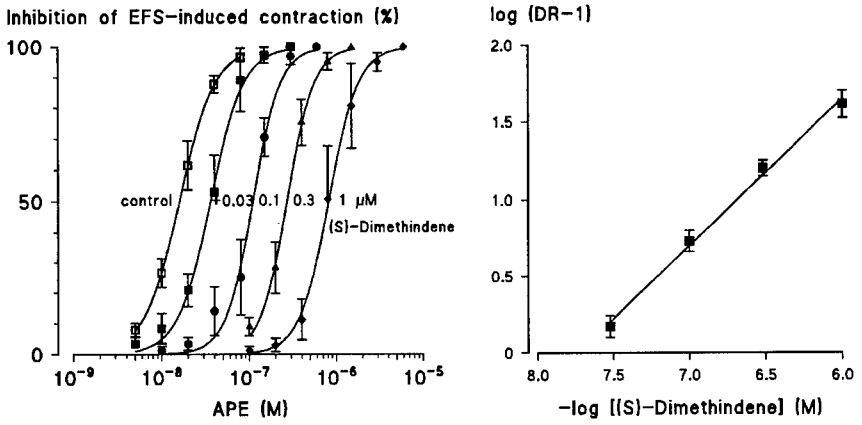

Fig. 2

Antagonism of increasing concentrations (solid symbols) of (S)-dimethindene on concentration-response curves for APE (control curve: open symbols) mediating inhibition of EFS-induced contractions in RAM (left panel). Numbers next to the concentration-response curves indicate the antagonist concentration. (S)-dimethindene was added 30 - 40 min before and kept throughout. Symbols represent the mean with S.E.M. as vertical lines from 3 - 6 experiments. The right panel shows the corresponding Schild plot, the slope of which (0.95 ± 0.10) was not significantly different from unity.

Modulation of neurogenic contractions via prejunctional muscarinic heteroreceptors

The potent and specific muscarinic agonist APE (0.02 - 10 μM) (25) did not affect the basal tension of the unstimulated preparation. In addition, contractile responses to exogenous NA were not affected by APE (10 μM), the pEC_{50} value for NA being 7.81 ± 0.08 in the absence of APE and 7.72 ± 0.11 in its presence. This suggests that a functional role of postsynaptic excitatory muscarinic receptors in RAM is unlikely. APE produced a complete, concentration-dependent and reversible inhibition of neurogenic contractions to sympathetic nerve stimulation at 1 Hz (Fig. 1 and 2), the pEC_{50} value being 7.74 ± 0.03. The inhibitory responses to APE were determined to be muscarinic in nature in that they were blocked by the muscarinic antagonists employed in the present study (see below). These data are consistent with the idea of prejunctional muscarinic receptors mediating heteroinhibition of NA release in the RAM.

All muscarinic antagonists employed shifted the APE concentration-response curves parallel to higher concentrations without any appreciable changes of basal tone or reduction of the maximum response; a representative plot for (S)-dimethindene is shown in Fig. 2 (left panel). Time-matched controls demonstrated that during the course of experiments there was no significant change in tissue sensitivity towards APE. For all the antagonists where Schild plots were obtained they were linear and the slopes of the plots were not significantly different from unity [illustrated for (S)-dimethindene in Fig. 2, right panel]. Thus all compounds behaved as competitive muscarinic receptor antagonists. However, it is worth noting that tripitramine displayed competitive antagonism of APE-induced inhibition of neurogenic contractions in RAM only at high concentrations (0.03 - 0.3 μM). The reason for the limited antagonism by tripitramine at low concentrations (1 - 10 nM) is unclear, but has also been observed previously at M_2 receptors in

guinea-pig atria and rabbit vas deferens (35). Further experiments are needed to clarify this issue. pA_2 values for the antagonists and slopes deduced from Schild analysis are listed in TABLE I.

TABLE I

Affinity Estimates (pA_2 Values; Antagonist Concentrations in Parentheses) and Slopes of Schild Plots for Muscarinic Antagonists Causing Inhibition of APE-Induced Decrease of Neurogenic Contractions to EFS in RAM. The Data are Presented as Means ± S.E.M.. Each Mean pA_2 Value was Obtained from n Determinations Including the Antagonist Concentrations Indicated. The Slopes of Schild Plots were not Significantly Different from Unity and therefore the Given pA_2 Values were Calculated after Constraining the Slopes to Unity

Antagonist	pA_2	μM	Slope	n
Pirenzepine	6.46 ± 0.04	(0.3; 1; 3; 10)	0.93 ± 0.08	14
AQ-RA 741	8.26 ± 0.05	(0.01; 0.03; 0.1; 0.3)	1.06 ± 0.08	13
Himbacine	8.04 ± 0.03	(0.01; 0.03; 0.1; 0.3)	0.98 ± 0.05	12
Tripitramine	9.10 ± 0.04	(0.03[a]; 0.1; 0.3)	0.90 ± 0.11	9
(S)-Dimethindene	7.69 ± 0.05	(0.03; 0.1; 0.3; 1.0)	0.95 ± 0.10	14
p-F-HHSiD	6.27 ± 0.28[b]	(1; 3[c])	---	8

[a] Lower concentrations showed no or limited antagonism.
[b] The apparent pA_2 value was determined from the individual dose-ratios according to the following equation: $pA_2 = \log (DR-1) - \log [B]$, where B = concentration of antagonist.
[c] Higher concentrations of p-F-HHSiD induced an increase of the basal tension and suppressed neurogenic contractions.

The rank order of antagonist potency found in the present study was: tripitramine > AQ-RA 741 ≥ himbacine ≥ (S)-dimethindene > pirenzepine ≥ p-F-HHSiD. *This rank order shows unequivocally that the prejunctional muscarinic receptors mediating heteroinhibition of NA release in RAM are of the M_2 subtype*. In detail, an excellent correlation was found (r = 0.99) by comparing the antagonist affinities from functional experiments in RAM with pK_i values obtained in radioligand binding assays at native and recombinant M_2 receptors (31, 36). Linear regression analysis of these highly correlated measures revealed slopes not significantly different from unity and intercepts not significantly different from zero. Much weaker correlations resulted when the antagonist potencies in RAM were compared with the binding affinities (31, 36) at M_1 (r = 0.13), M_3 (r = -0.44), M_4 (r = 0.63) and M_5 receptors (r = 0.02). As far as comparisons of functional affinities of the antagonists employed in the present study are concerned, there is a remarkable agreement in the antimuscarinic potencies obtained in RAM and at M_2 receptors in guinea-pig atria (r = 0.99) and rabbit vas deferens (r = 0.98) (37 - 41).

Conclusions

The present study demonstrates that EFS-induced contractions in RAM (in the presence of L-NOARG) are caused by exocytotic release of NA from sympathetic nerves acting at postjunctio-

nal α_1-adrenoceptors. An involvement of ATP as co-transmitter or a pre- or postjunctional modulation of the neurogenic responses by endogenous ATP (from extraneuronal sources), prostanoids and acetylcholine can be excluded. The evoked release of NA is controlled by prejunctional inhibitory muscarinic receptors. Examination of these heteroreceptors by the use of a series of subtype-prefering key muscarinic antagonists provided strong evidence that they are of the M_2 subtype. Any contribution of a second prejunctional muscarinic receptor is highly unlikely, since no deviations from competitive behaviour of the antagonists employed were observed. Postjunctional excitatory muscarinic receptors could not be identified in RAM.

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FUNCTION, SIGNAL TRANSDUCTION MECHANISMS AND PLASTICITY OF PRESYNAPTIC MUSCARINIC RECEPTORS IN THE URINARY BLADDER

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Summary

Presynaptic M_1 muscarinic receptors on parasympathetic nerve terminals in rat urinary bladder strips are involved in an autofacilitatory mechanism that markedly enhances acetylcholine release during continuous electrical field stimulation. The facilitatory muscarinic mechanism is dependent upon a PKC mediated second messenger pathway and influx of extracellular Ca^{2+} into the parasympathetic nerve terminals via L and N-type Ca^{2+} channels. Prejunctional muscarinic facilitation has also been detected in human bladders. The muscarinic facilitatory mechanism is upregulated in hyperactive bladders from chronic spinal cord transected rats; and the facilitation in these preparations is primarily mediated by M_3 muscarinic receptors. Presynaptic muscarinic receptors represent a new target for pharmacological treatment of bladder hyperactivity. If presynaptic facilitation is restricted to the bladder and not present in other tissues then drugs acting at this site might be expected to exhibit uroselectivity.

Key Words: M_1 muscarinic receptors, presynaptic facilitation, acetylcholine release, urinary bladder

1. Introduction

The urinary bladder receives an innervation from both the parasympathetic and sympathetic divisions of the autonomic nervous system. The sacral parasympathetic outflow provides the major excitatory input to the detrusor muscle; whereas the lumbar sympathetic nerves provide an excitatory input to the bladder neck and an inhibitory input to the detrusor. Parasympathetic excitatory nerves release acetylcholine as well as the non-cholinergic transmitter, ATP. The relative importance of cholinergic and purinergic excitatory mechanisms in the bladder varies between species; however, in humans cholinergic excitatory transmission mediated by muscarinic receptors plays the major role.

Muscarinic receptors are present in both the smooth muscle and in the parasympathetic and sympathetic nerve endings. In the bladder smooth muscle two types of postjunctional muscarinic receptors have been identified in various species. In the rat a high density of M_2 and a lower density of M_3 receptors have been detected (1). The M_3 receptors mediate the contractile response to neurally released ACh; whereas M_2 receptors may inhibit adenylate cyclase and therefore suppress β-adrenoceptor mediated relaxation (2). There is also evidence for a significant role of M_3 muscarinic receptors in the contractions of human bladders (3). Thus postjunctional muscarinic excitatory receptors in the bladder are important targets for pharmacologic treatment of bladder hyperactivity.
Presynaptic muscarinic receptors have attracted less attention as possible targets for drug therapy because in various organs (heart, ileum, salivary glands) (4) including the bladder (5, 6) it has usually

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been found that these receptors mediate an autoinhibitory control over ACh release. In the urinary bladder, prejunctional inhibitory mechanisms are mediated by M_4 receptors (7, 8). However, recent studies have also identified muscarinic facilitatory mechanisms in both parasympathetic and sympathetic nerves in the urinary bladder (9). These mechanisms which have some unusual properties may provide new targets for pharmacotherapy of hyperactive bladder disorders. This paper will review experiments conducted on rat, cat and human bladders that have evaluated various features of presynaptic modulation including: stimulation parameters, species differences, signal transduction mechanisms and plasticity induced by pathology.

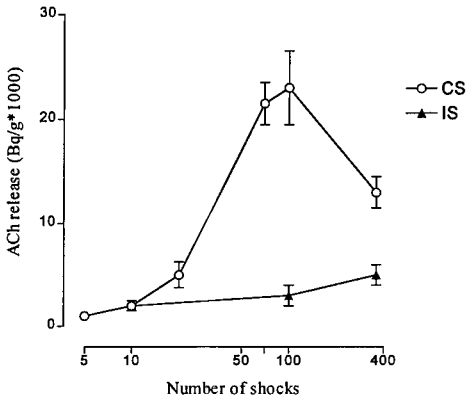

Fig. 1.
Effects of number of shocks on the release of radio-labeled ^{3}H-ACh from the rat urinary bladder. The number of shocks (abscissa) was plotted against the released amount of ACh expressed as disintegration/sec/g (Bq/g). Note that continuous stimulation (CS) released significantly higher amounts of ACh than intermittent stimulation (IS) at a frequency of 10 Hz.

2. Activation of inhibitory and facilitatory muscarinic prejunctional receptors.

ACh release in rat urinary bladder strips is influenced by various stimulation parameters including frequency, pattern and duration of stimulation. During intermittent field stimulation (IS), consisting of short trains (10 shocks) separated by 5 sec quiescent periods, presynaptic facilitatory mechanisms were not activated and the duration of stimulation (5-360 shocks) had little effect on the total ACh output (non-facilitatory stimulation). Therefore the output per volley decreased as the number of shocks increased. On the other hand, with continuous stimulation (CS) ACh release per volley markedly increased as the number of shocks was increased from 5 to 70 (facilitatory stimulation) (Fig 1). The facilitation of ACh release was blocked by atropine or pirenzepine indicating that it was mediated by M_1 muscarinic receptors (9).The contractions of rat bladder strips were also enhanced by CS; and this enhancement was reduced by low concentrations of pirenzepine (10). The lower range of CS (10 Hz, 100 shocks) which mimics the physiological firing rates of bladder parasympathetic neurons (11) and which produces maximal bladder contractions elicits a marked recruitment of ACh release. This recruitment does not occur in cardiac atrial tissue, the ileum (12,13), or the trachea (14).

In the bladder both the CS- and the IS-evoked release were considerably enhanced by treatment with eserine (6). The CS- and eserine-induced facilitation are likely mediated by a similar muscarinic mechanism since both were blocked by atropine or pirenzepine. The marked facilitatory effect of eserine on CS-evoked ACh release suggests that M_1 receptors are not fully activated by CS in the absence of eserine. This facilitatory effect of eserine on ACh release was not detected in other tissues such as ileum (19) and heart (Fig. 3).

In the urinary bladder the presynaptic inhibitory effect mediated by M_4 receptors does not appear to be expressed during M_1 facilitation since the facilitated release was inhibited to the same extent by either the non-selective muscarinic blocker, atropine, which blocks M_1 and M_4 receptors or the M_1 selective agent, pirenzepine (9). If M_4 inhibition were active it would be expected that atropine would block the inhibition and produce less depression of ACh release than pirenzepine. Since this does not occur we conclude that during CS, M_4 mechanisms are turned off. This raises the possibility that presynaptic muscarinic receptor mechanisms in the urinary bladder are plastic and are capable of functioning in either facilitatory (supposedly M_1) or inhibitory (supposedly M_4) modes depending on the junctional ACh level.

It also seems likely that the M_1 facilitation is modulated by an inhibitory mechanism which is

unmasked by intermittent stimulation. For example, a single short train of stimuli induced M_1 facilitation; however, multiple short trains suppressed the facilitation (9). Presumably, periods of quiescence, which occur during IS, allow for the early expression of inhibition, whereas, CS initially blocks or delays the development of inhibition which eventually appears with longer trains of stimulation (360 shocks) (9). In accordance with this proposal 360 shocks releases less ACh than 100 shocks (Fig. 1) suggesting that the release of ACh is not complete by the end of a 100 shock stimulus train and that extending the train duration can inhibit the delayed release evoked by the initial volleys in the stimulus train.

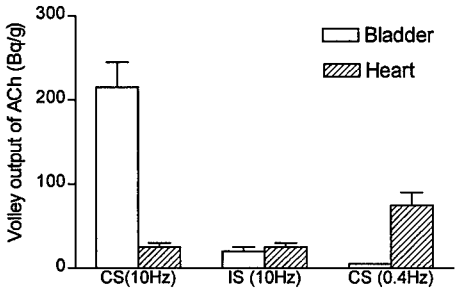

Fig. 2

Tissue dependence of the muscarinic facilitation of ACh release. The ordinate shows the volley output of ACh (Bq/g) (ACh release for one stimulus pulse). Note that CS with 10Hz caused muscarinic facilitation only in the bladder, IS had the same effect in both tissues, whereas, CS with 0.4Hz (100 shocks) caused higher release of ACh in the heart than in the bladder.

Interestingly muscarinic receptors at adrenergic nerve terminals in the rat bladder show the same stimulation dependent functions as those in cholinergic terminals. When bladder strips are stimulated by low frequency stimulation (2 Hz) the inhibitory M_4 receptors are activated and as a result NE release can be enhanced by atropine (15). However, CS induced high release of ACh activates the presynaptic facilitatory M_1 receptors which enhance NE release. Under these circumstances atropine suppresses norepinephrine release (16). These results indicate that ACh released from cholinergic terminals can interact heterosynaptically with adjacent adrenergic terminals.

3. Tissue dependence of muscarinic facilitation

The muscarinic facilitatory mechanism distinguishes cholinergic nerve terminals in the bladder from terminals in other tissues, such as heart (17, 18) (Fig. 2) , intestine (19) and striated muscle (20), which exhibit different types of presynaptic modulatory mechanisms. In the urinary bladder stimulation with high frequencies released more ACh per impulse than low frequencies. This is opposite to the effect of high frequency stimulation on ACh release in cardiac (Fig. 2) as well as in ileal tissues (12, 13, 21).

On the other hand, at cholinergic terminals in other organs atropine enhances the release of ACh by blocking inhibitory M_2 or M_4 presynaptic muscarinic receptors and this effect is more prominent in the presence of eserine (13, 19, 22). The inhibitory effect of eserine is attributable to a reduction in ACh metabolism leading to an increase in its synaptic concentration, which results in a more effective activation of the presynaptic inhibitory muscarinic receptors.

4. Species dependence of muscarinic facilitation:

After the identification of prejunctional muscarinic facilitation in the rat, studies in another laboratory have provided evidence for a similar mechanism in the rabbit (23). Presynaptic facilitation has also been detected at the parasympathetic nerve terminals in the human bladder. In human tissue the facilitation also seems to be mediated by M_1 receptors (24). However, in cat bladders presynaptic facilitation was not detected (Somogyi, unpublished). On the other hand, in the cat a prominent frequency dependent, presynaptic facilitation occurs in the bladder parasympathetic ganglia (25). This facilitation is not mediated by muscarinic receptors but does serve the same function to amplify efferent activity passing from the spinal cord to the bladder.

5. Signal transduction mechanisms underlying muscarinic facilitation:

a) the role of protein kinase C (PKC):

The phosphatidylinositol-PKC cascade is known to be one signal transduction pathway linked to M_1 muscarinic receptors (26). In addition, activation of PKC can facilitate the release of various transmitters including ACh in the peripheral and central nervous system (27). In the urinary bladder

activation of PKC by phorbol dibutyrate (PDB) enhanced ACh release (Fig.3) in a concentration dependent manner. However, an inactive phorbol ester, 4α-phorbol didecanoate, was ineffective (16). Conversely, the PKC blocker, H-7 (28, 29) suppressed the facilitation of ACh release induced by continuous stimulation (Fig. 3) or eserine, suggesting an important role of protein phosphorylation in the facilitatory mechanism. H-7 did not block the non-facilitated release of ACh induced by intermittent stimulation indicating that the PKC system only participates in transmitter release under facilitatory conditions (16). However, H-7, like other PKC blockers, is not specific for PKC, and can also block other kinases, such as PKA (29). Nevertheless, the specific PKA inhibitor, HA-1004 which does not affect PKC (30, 31) did not affect the facilitated release of ACh in the urinary bladder (16). In addition the stable analogue of cAMP, 8-Br cAMP, which activates PKA, did not alter ACh release indicating that PKA is not involved in the M_1 facilitation of ACh release in the urinary bladder (16). Thus it is reasonable to conclude that the effect of H-7 was mediated by an action on PKC and not on PKA. The crucial role of PKC in M_1 facilitation is further supported by the fact that down regulation of the enzyme by pretreatment of bladder strips with a high concentration of a phorbol ester (32) inhibited the facilitated release of ACh (16).

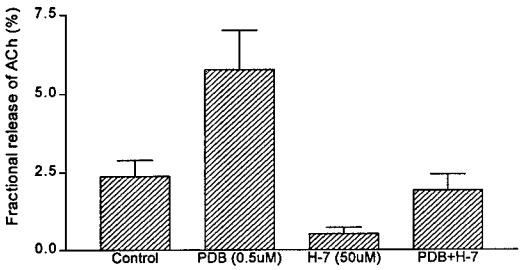

Fig. 3.
The effect of activation or inhibition of PKC on the facilitated release of ACh evoked by CS (10Hz/100shocks). The release of ACh is expressed as fractional release (the release of labeled ACh expressed as percent of the tissue radioactivity). PDB significantly enhanced ACh release; whereas H -7 inhibited the facilitated release. The PDB induced enhancement of the release was reduced by H-7 to the control level

However, the facilitation of ACh release initiated by PKC stimulation was blocked by atropine (16). This unexpected effect of atropine was quantitatively similar to its effect on CS or eserine-induced facilitation of ACh release, which would be attributed to block of M_1 receptors on cholinergic terminals. Thus, facilitation induced by PKC activation must also require functional M_1 receptors even if it is initiated down-stream from the receptors. Cholinergic facilitation therefore appears to be a self-amplifying process, in which the released ACh feeds back positively onto the M_1 muscarinic receptors to further activate PKC and release more ACh. This is consistent with the data indicating that the M_1 facilitatory mechanism is relatively slow in the onset, and requires a relatively long time to reach its maximum (9).

b. The role of calcium channels:

Facilitated ACh release in the urinary bladder was increased in a graded manner as Ca^{2+} concentration in the bathing solution was increased from 1 to 2.5 mM, indicating that extracellular Ca^{2+} concentration has an important role in the M_1 presynaptic facilitatory mechanism (9). The Ca^{2+} influx which contributes to transmitter release from parasympathetic nerve terminals of the urinary bladder occurs via several types of Ca^{2+} channels. The relative importance of these channels varies under different experimental conditions Under non-facilitatory stimulation conditions blockade of N channels inhibits ACh release in mouse and rat bladder (33, 34, 35). Nifedipine, an L channel blocker, did not significantly reduce release of ACh under non-facilitatory conditions. However, P and Q channel blockers are effective in depressing excitatory transmission in the mouse bladder when administered after blockade of N channels (34). Thus it would appear that under non-facilitatory conditions, N channels play a prominent role, but that P and Q type channels can maintain a lower level of transmitter release in the absence of N channels. A similar role for multiple types of Ca^{2+} channels has been demonstrated at other sites in the peripheral and central nervous system (36, 37).

In our studies in the rat bladder, enhancement of transmission by eserine, PDB or CS, all of which activate muscarinic presynaptic facilitatory mechanisms (9), unmasked a prominent contribution of L channels to the release of ACh (35) (Fig. 4). The enhanced release (range: 2.4 to 11.8 fold) of ACh induced by these three facilitating conditions was significantly reduced (range: 31 to 83%) by

nifedipine, indicating a role for L type Ca^{2+} channels. In every instance in which nifedipine was effective in reducing the facilitation, atropine also reduced or completely blocked the facilitation, demonstrating an involvement of muscarinic receptors and indicating that muscarinic facilitation of ACh release in the bladder is linked with L channel mechanisms.

The facilitation of ACh release induced by PKC activation was not completely abolished by nifedipine (67%) nor by combined treatment with nifedipine and ω-conotoxin (ω-CgTX) (79%) an N channel blocker. The partial inhibition of the PKC-induced facilitation by L and N-type Ca^{2+} channel blockers suggests that the PKC activation facilitates ACh release by mechanisms in addition to those dependent on L and N type Ca^{2+} channels; possibly involving P/Q type channels (34).

Based on the effectiveness of nifedepine, it appears that L channels play a major role in the facilitation of ACh release by eserine (70% and 83% inhibition of release) as well as in the PDB enhancement of ACh release (67% inhibition of release), but a lesser role (31% to 43% inhibition of release) in the facilitation evoked by CS (Fig 4.). This effectiveness correlates with the magnitude of the facilitation and presumably the level of muscarinic receptor activation; ie., eserine produces the largest facilitation (e.g. 17 fold increase in ACh release) and CS produces a much smaller facilitation (e.g. 5.5 fold increase in ACh release)(35).

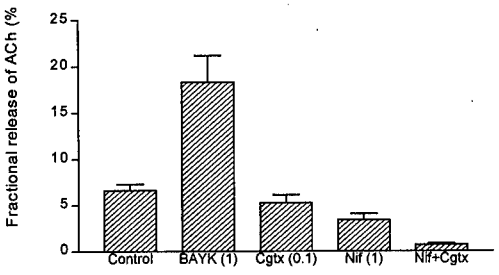

Fig. 4.
The effect of the L-type Ca^{2+} channel activation by BAY-K 8644 and L and N-type channel blockade on the facilitated release of ACh in the urinary bladder. Note that the L-type channel blocker nifedipine significantly inhibited ACh release; whereas the N-type channel blocker ω-conotoxin (ω-Cgtx) alone did not block the release. A combination of nifedipine and ω-CgTX markedly suppressed release.

The remainder of the facilitation produced during CS appears to be due to activation of N type Ca^{2+} channels since combined treatment with nifedipine and a low concentration (20nM) of ω-CgTX produced an almost complete block of ACh release (86-91% inhibition) (Fig. 4). However, the administration of a higher concentration (100nM) of ω-CgTX alone did not alter CS facilitation of ACh release. These data indicate that at cholinergic terminals Ca^{2+} influx through L channels is sufficient to produce a maximal CS-facilitation. However, after blockade of L-channels, Ca^{2+} influx through the N- channels can maintain approximately 50% of the muscarinic facilitation (35) .

The effectiveness of L channels to facilitate transmitter release in the urinary bladder was also demonstrated with BAY-K 8664, an agent that enhances the opening time of L channels (Fig. 4) (16). During IS, BAY-K 8664 elicited a large increase in ACh release (5 fold increase) This is consistent with the more prominent role of L channels in the CS-induced facilitation of cholinergic transmission. The effects of BAY-K 8664 were also reduced by atropine indicating that the increased concentrations of ACh after Ca^{2+} channel activation are able to activate muscarinic autoreceptors to augment the direct facilitatory action of BAY-K 8664 on L channels.

Because the facilitation of ACh release depends upon activation of muscarinic receptors, L channels and PKC, it is tempting to speculate that muscarinic receptors act through a G protein to stimulate phospholipase C which then increases the levels of diacylglycerol leading to the activation of PKC and the subsequent phosphorylation and facilitation of the L channels (Fig. 5). The final step in this hypothetical pathway receives support from other studies showing that: a) activators of PKC facilitate transmission at various sites (16), b) PKC enhances L channel currents (38) and c) Ca^{2+} channel subunits can be phosphorylated by PKC (38).

In contrast to the results obtained at nerve terminals in the urinary bladder, muscarinic receptor mediated enhancement of Ca^{2+} channels is not a common finding at other sites in the nervous system.

Indeed most studies have shown that muscarinic agonists inhibit Ca^{2+} channels (36, 38; 40) and/or inhibit transmitter release (41, 42). Thus bladder efferent pathways seem to be unusual in this regard. Consistent with the present findings it has recently been reported that muscarinic agonists can enhance L type Ca^{2+} channel currents in the neurons of the major pelvic ganglia (43). A subpopulation of these neurons provide the excitatory innervation to the bladder. This effect contrasts with the well known inhibitory effect of muscarinic agonists on N and L type Ca^{2+} currents in sympathetic neurons from the superior cervical ganglion (40).

6. Plasticity of the presynaptic muscarinic receptors in the bladder:

The urinary bladder in the rat undergoes a profound morphological and functional plasticity after spinal cord injury. After the initial phase of spinal shock, a spinal reflex pathway emerges that mediates automatic micturition and bladder hyperreflexia (44). In addition, coordination between the external urethral sphincter and the bladder is lost resulting in functional bladder outlet obstruction and an increase in bladder mass (45) which is accompanied by changes in the properties of the bladder afferent (46) and efferent pathways (47). The above described changes in bladder function in spinal cord transected (SCT) rats were also accompanied by a significant change in ACh release from bladder nerves (48). These changes in ACh release were detected using a range of stimulation frequencies in bladder strips from neurally intact (NI) and SCT rats. In NI rats ACh release was not facilitated with CS at 2 Hz, 100 shocks but some facilitation occurred at 5 Hz and more prominently at 10 and 40 Hz indicating a positive correlation between the degree of facilitation and the stimulation frequency. However, when the frequency dependence of ACh release was analyzed in the SCT bladder strips, it was noted that the release was facilitated at 2 Hz and reached a maximum at 10 Hz indicating a left shift of the frequency response curve (Fig 6).

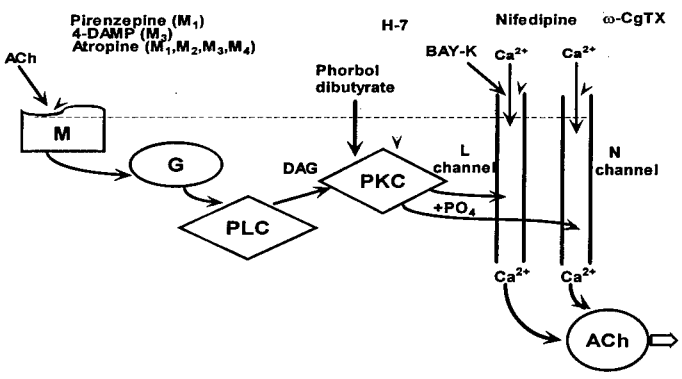

Fig. 5

Schematic diagram of cholinergic terminals in the bladder showing the likely intracellular signalling mechanisms underlying the facilitation of transmitter release by muscarinic receptor (M) activation. G: G protein; PLC: phospholipase C; PKC: protein kinase C. Agonists: solid arrows; antagonists: dotted arrows.

Since facilitation of ACh release is inhibited by atropine, atropine can be used to determine the degree of presynaptic M_1 facilitation of transmitter release. The release of ACh at 2 and 5 Hz stimulation in NI bladders was unchanged in the presence of atropine indicating that there was minimal M_1 facilitation under these conditions; whereas ACh release was reduced by atropine at 10 and 40 Hz indicating that the release was facilitated at these frequencies. However, atropine suppressed ACh release in SCT bladders at 2 and 5 Hz indicating that M_1 facilitation must occur at a lower frequency range in SCT bladders strips (48) (Fig. 6).

The possible explanation for the left shift of the frequency response curves in SCT bladder strips is that the presynaptic facilitatory muscarinic receptors or the signal transduction mechanisms are upregulated in SCT bladders and thus the facilitation occurs in a lower frequency range (2-5 Hz), where normally there is no or very slight facilitation of ACh release. As a result there is a more intense muscarinic facilitation of ACh release at 2-5 Hz stimulation and a more prominent effect of atropine to inhibit ACh release in SCT bladder strips than in NI bladder strips. An alternative explanation is that presynaptic inhibitory muscarinic receptors are down-regulated in SCT bladders. The down regulation of presynaptic M_2 muscarinic receptors under pathological circumstances has been previously described in the lung (49).

Interestingly the subtype of the facilitatory muscarinic receptors also appeared to be changed in SCT bladders. This conclusion is based on the relative effectiveness of the M_1 receptor selective blocker, pirenzepine and the M_3 selective blocker, 4-DAMP to suppress the facilitation. While in NI bladders the facilitation was blocked by pirenzepine (10-20 nM), the same concentration was ineffective in SCT bladders. Conversely, in SCT bladders 4-DAMP was effective in a much lower concentration(10 nM) than in NI bladders, indicating that M_3 rather than M_1 muscarinic receptors mediate the facilitatory release in SCT bladder preparations (50).

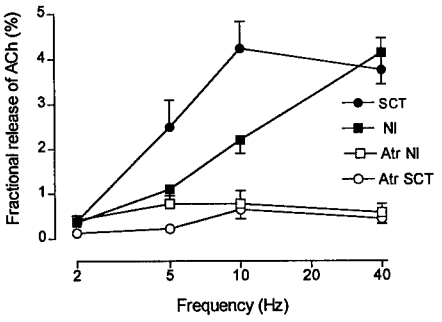

Fig. 6.
Frequency dependence of ACh release from neurally intact (NI) and spinal cord transected (SCT) rats. The release of ACh was evoked by 100 shocks delivered at different frequencies, The release values in NI and SCT bladders are significantly different from each other ($P<0.05$) at 5 and 10 Hz. Atropine significantly reduced ACh release in NI bladder strips at 10Hz and 40 Hz stimulation, however in SCT bladders atropine reduced the release at all frequencies.

7. Conclusions:

The efferent signals passing to the bladder from the spinal cord are amplified by facilitatory mechanisms either at the level of the bladder ganglia (cats) or at the parasympathetic nerve terminals (rat, rabbit, human). The latter facilitation is mediated by presynaptic M_1 muscarinic receptors. This facilitatory mechanism is upregulated after spinal cord injury and appears to be mediated by a different receptor subtype, ie., M_3 instead of M_1. Muscarinic receptors are important targets for the medical treatment of bladder hyperactivity. However, a major side effect of antimuscarinic agents is dry mouth due to blockade of M_3 receptors in the salivary glands. If presynaptic muscarinic facilitatory receptors are restricted to the bladder and not present in the salivary glands, then it is possible that drugs targeting these receptors might exhibit some degree of uroselectivity and reduce bladder hyperactivity with fewer side effects.

Acknowledgments

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MUSCARINIC RECEPTOR SUBTYPES MODULATING SMOOTH MUSCLE CONTRACTILITY IN THE URINARY BLADDER

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Summary

Normal physiological voiding as well as generation of abnormal bladder contractions in diseased states is critically dependent on acetylcholine-induced stimulation of contractile muscarinic receptors on the smooth muscle (detrusor) of the urinary bladder. Muscarinic receptor antagonists are efficacious in treating the symptoms of bladder hyperactivity, such as urge incontinence, although the usefulness of available drugs is limited by undesirable side-effects. Detrusor smooth muscle is endowed principally with M_2 and M_3 muscarinic receptors with the former predominating in number. M_3 muscarinic receptors, coupled to stimulation of phosphoinositide turnover, mediate the direct contractile effects of acetylcholine in the detrusor. Emerging evidence suggests that M_2 muscarinic receptors, via inhibition of adenylyl cyclase, cause smooth muscle contraction indirectly by inhibiting sympathetically (β-adrenoceptor)-mediated relaxation. In certain diseased states, M_2 receptors may also contribute to direct smooth muscle contraction. Other contractile mechanisms involving M_2 muscarinic receptors, such as activation of a non-specific cationic channel and inactivation of potassium channels, may also be operative in the bladder and requires further investigation. From a therapeutic standpoint, combined blockade of M_2 and M_3 muscarinic receptors would seem to be ideal since this approach would evoke complete inhibition of cholinergically-evoked smooth muscle contractions. However, if either the M_2 or M_3 receptor assumes a greater pathophysiological role in disease states, then selective antagonism of only one of the two receptors may be the more rational approach. The ultimate therapeutic strategy is also influenced by the extent to which pre-junctional M_1 facilitatory and M_2 inhibitory muscarinic receptors regulate acetylcholine release and also which subtypes mediate the undesirable effects of muscarinic receptor blockade such as dry mouth. Finally, the consequence of muscarinic receptor blockade in the central nervous system on the micturition reflex, an issue which is poorly studied and seldom taken into consideration, should not be ignored.

Key Words: **muscarinic receptors, bladder, detrusor, incontinence, M_2 muscarinic receptors, M_3 muscarinic receptors**

The process of micturition is critically dependent on the operation of a spinal-bulbospinal reflex which comprises afferent (sensory) and efferent (motor) peripheral pathways which are integrated and co-ordinated at spinal and supra-spinal centers (1). The lumbosacral parasympathetic outflow provides the excitatory motor input to the urinary bladder smooth

muscle (detrusor). Activation of the parasympathetic system is the major pathway by which bladder contraction, and thus voiding, is achieved in man and primates (2). During the filling phase, the parasympathetic drive is normally supressed thereby facilitating relaxation and low pressure urine storage. However, in patients with various forms of detrusor hyperactivity, it is thought that excessive cholinergic input accompanied by muscarinic receptor supersensitivity contributes to the generation of involuntary detrusor contractions resulting in urinary incontinence (3). This underlies the established usefulness of muscarinic receptor antagonists, such as oxybutynin, in the treatment of urinary incontinence (3). The clinical utility of available muscarinic antagonists is limited by their anti-cholinergic side-effects, mainly dry mouth. Given the heterogeneity of muscarinic receptors, it may be possible to develop compounds with receptor subtype specificity and greater selectivity for the bladder, provided one has a precise understanding of the localization, pharmacology and functional role of the different receptors in the urinary bladder. The primary objective of this article is to review the current knowledge in this area from a multidisciplinary standpoint. The reader is also referred to recent reviews on muscarinic receptors which cover additional aspects of smooth muscle pharmacology including other genitourinary tissues (4, 5, 6).

Molecular and radioligand binding studies

The techniques of northern blot hybridization, reverse transcriptase polymerase chain reaction (RT-PCR) and immunoprecipitation have yielded useful knowledge on the identity of the muscarinic receptors in the bladder at the mRNA/protein level. Northern blot hybridization analyses using receptor specific probes have revealed the presence of only m_2 and m_3 mRNA in the rat (7, 8) and pig (7) bladder. In semi-quantitative RT-PCR experiments, the presence of only m_2 and m_3 transcripts (1:1) could be detected in the human bladder (9). In contrast, studies in the rat bladder have found m_1, m_2, m_3 and m_4 transcripts (10). Using antisera directed against each of the five muscarinic subtypes, only m_2 and m_3 subtypes could be precipitated from rat (11, 12), rabbit (12), guinea-pig (12) and human (12) bladder membranes. Furthermore, it was shown that the m_2: m_3 ratio was 9:1 in the rat bladder and 3:1 in the bladders of other species studied.

Saturation binding studies using non-selective radioligands ([^{3}H]-N-methyl scopolamine, [^{3}H]-quinuclidinyl benzylate) have established that the urinary bladder, regardless of species, is enriched with muscarinic binding sites whose density has been reported to be in the range of 80 to 450 fmoles/mg of protein (13 – 18). High affinity specific [^{3}H]-pirenzepine binding sites have reported to be either absent (19) or present in low density (20) in the urinary bladder excluding a major M_1 receptor population in this tissue. The existence of multiple binding sites in the rat urinary bladder was suggested by Monferinni et al. (17) who demonstrated that AF-DX-116 competed for specific muscarinic binding sites labelled by [^{3}H]-N-methyl scopolamine in a heterogenous manner consistent with high affinity (possibly M_2) and low affinity (possibly M_3) sites in the ratio of approximately 9: 1. However, studies in the rabbit (21), guinea-pig (22), pig (18) and human (18, 23) bladder have indicated the almost exclusive presence of M_2 receptors. One study in the human bladder has, surprisingly, shown the predominance of M_3 receptors (20). While some of these discrepant findings may be species related, a more plausible explanation is that the experimental conditions (ligand and ionic strength of buffer used) in some of these reported studies were not optimal for allowing discrimination between M_2 and M_3 muscarinic receptors. It is well know that the ionic strength of buffer in radioligand binding studies affects the affinity estimates of ligands especially those which display M_2 receptor selectivity (24). Indeed, competition binding studies conducted in Tris-EDTA buffer and using tripitramine (25), a ligand that displays >100-fold selectivity for M_2 over M_3 receptors have clearly shown a dominant (60 – 80%) M_2 population (high affinity for

triptramine, pK_i = 9.4) and a smaller (20 – 40%) M_3 population (low affinity for triptramine, pK_i = 7.1) in the urinary bladder of rat (26) and man (Fig. 1A & B).

Overall, the results from radioligand binding studies closely parallel the findings from immmunological studies which have also shown the greater abundance of the m_2 receptor protein compared to the m_3 receptor protein. Few studies have sought to investigate the regional distribution of M_2 and M_3 receptors in the bladder body. This avenue of research may be worthy of pursuit since the bladder dome and base have different functions during the voiding phase.

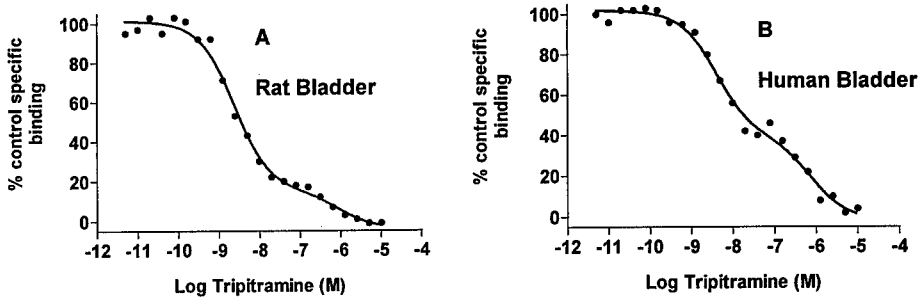

FIG. 1

Displacement of [^{3}H]-N-methyl scopolamine specific binding by tripitramine in membranes from rat (A) and human (B) bladder. Membranes were incubated with 1 nM [^{3}H]-N-methyl scopolamine and various concentrations of tripitramine in 50 mM Tris-EDTA buffer for 1.5 – 2 h at 25^{0}C. Non-specific binding was defined with 1 μM atropine. The data are consistent with a heterogenous population of two sites: one with a high affinity for tripitramine ($pK_{i\,(H)}$ = 9.3 – 9.5) and the other with a low affinity for tripitramine ($pK_{i\,(L)}$ = 7.1). The two sites, corresponding to M_2 and M_3 receptors, are present in the ratio of approximately 4:1 in the rat bladder and 3:2 in the human bladder.

Receptor-Effector mechanisms

Co-immunoprecipitation studies in the rat bladder (12) have shown that the m_2 and m_3 receptor proteins couple to the Gi and $G_{q/11}$ family of guanine nucleotide proteins, respectively, activation of which would be expected to result in inhibition of adenylyl cyclase and stimulation of phospholipase C, respectively.

Muscarinic receptor stimulation induces phosphoinositide hydrolysis in the guinea-pig (27), rat (8) and human (19, 28) urinary bladder. The elevated levels of inositol phosphates presumably causes release of intracellular calcium. The receptor mediating the phosphoinositide-stimulatory response, which undergoes rapid and homologous desensitization (29), has been pharmacologically characterized in cultured human detrusor cells (Fig. 2A) and shown to display a high affinity (pA_2) for 4-DAMP (9.0), an intermediate affinity for p-F-HHSiD (7.4) and a low affinity for pirenzepine (6.9) and methoctramine (6.3); these data being consistent with the involvement of M_3 muscarinic receptors (30).

Muscarinic agonists have been shown to inhibit adenylate cyclase in the rabbit (19), guinea-pig (27) and human (19) bladder. This mechanism, under physiological conditions, may serve to oppose β-adrenoceptor-mediated augmentation of cAMP levels in bladder smooth muscle (see

below). Daniels et al. (31) have recently characterized the pharmacology of this response in cultured human detrusor cells in which carbachol produces robust inhibition of forskolin-stimulated cAMP accumulation (Fig. 2B). The inhibitory response to carbachol was concentration-dependently inhibited by subtype-selective antagonists yielding the following affinity estimates (pK_B): pirenzepine (< 6.0), darifenacin (< 6.8), AQ-RA-741 (8.7), tripitramine (8.9), PD 102807 (6.1), MT3 (< 5.0), himbacine (8.2); these values equate with the pharmacologically defined M_2 receptor.

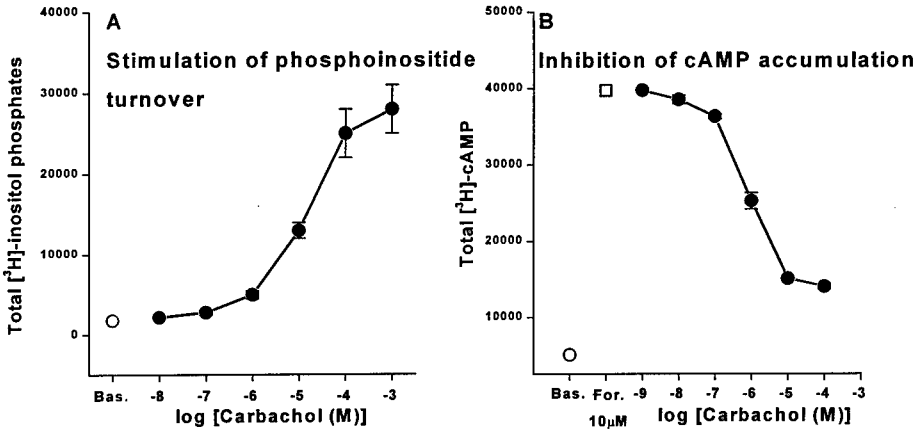

FIG. 2

Muscarinic M_3-receptor mediated stimulation of phosphoinositide turnover (A) and M_2-receptor-mediated suppression of forskolin (For.)-stimulated c-AMP accumulation (B) in cultured human detrusor cells. Data for (A) and (B) are taken from references (30) and (31), respectively.

M_2 receptor-mediated signaling mechanisms, apart from inhibition of adenylate cyclase, have been demonstrated in non-bladder smooth muscle and may also be operative in the urinary bladder. For example, in the ileum of the guinea-pig, M_2-receptor agonism causes opening of non-selective cation channels resulting in depolarisation (32) and, consequently, influx of calcium. A similar mechanism has, thus far, not been demonstrated in the detrusor. In certain tissues, such as canine colon, M_2 muscarinic receptor activation causes direct inhibition of conductance through potassium channels (33). Interestingly, two studies have demonstrated an inhibitory effect of carbachol on K_{ATP} (34) and Ca^{2+} activated K-channels (35) in bladder smooth muscle from the guinea-pig and rat, respectively, although the pharmacology of these responses were not thoroughly investigated. Lastly, M_2 receptors in the bladder could potentially activate Rho proteins, as demonstrated in airway smooth muscle (36), and cause Ca^{2+} sensitization of the contractile machinery.

Functional studies

Pharmacological antagonist characterization of muscarinic receptors mediating direct contraction of detrusor muscle in rat (12, 37, 38) rabbit (39), mouse (40) guinea-pig (27), monkey (41) and human (42) bladder suggests the singular involvement of M_3 receptors (Fig. 3A). This raises questions about the functional role of the M_2 receptor, which predominates in every species studied.

Emerging evidence, however, appears to shed light on the role of M_2 receptors in the bladder. Under conditions in which M_3 receptors are selectively alkylated, M_2 receptors were shown to reverse β-adrenoceptor-mediated relaxation (referred to as indirect contraction or re-contraction) (38, 43) (Fig. 3B). In anesthetized rats, methoctramine, a selective M_2 receptor antagonist, potently inhibits reflex volume-induced bladder contractions (38). Furthermore, pre-treatment with propranolol decreased the inhibitory potency of methoctramine. Also, the inhibitory potency of several antagonists correlated most favorably with their affinity for M_2 muscarinic receptors. These findings suggest that the role of M_2 receptors in the bladder may be

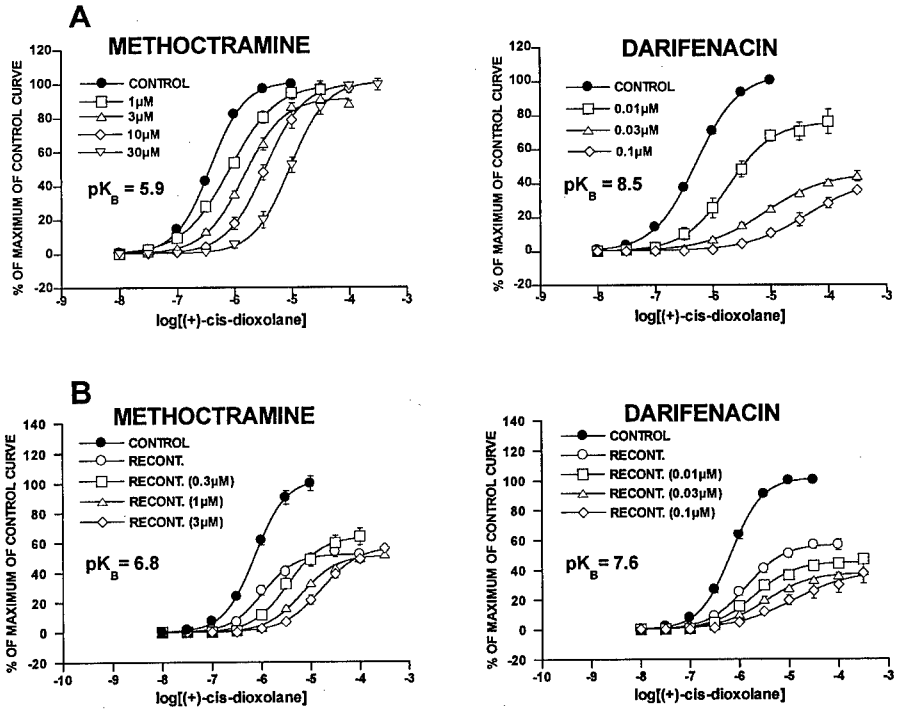

FIG. 3

Demonstration of direct (M_3) and indirect (M_2) muscarinic contractile mechanisms in the rat isolated bladder. A: Under standard assay conditions, (+)-cis-dioxolane produces concentration-dependent direct contractile responses which are antagonized by methoctramine and darifenacin with affinity estimates (5.9 and 8.5, respectively) which are consistent with the singular involvement of M_3 muscarinic receptors. B: Under conditions in which M_3 receptors are preferentially alkylated (exposure to 4-DAMP mustard (40 nM, 1h) in the presence of methoctramine (0.3 μM)) and adenylyl cyclase is stimulated (isoproterenol-induced relaxation of KCl-precontracted tissues)), (+)-cis-dioxolane produces concentration-dependent recontractions (RECONT.) which are antagonized by methoctramine and darifenacin with affinity estimates (6.8 and 7.6, respectively) that are consistent with the involvement of both M_2 and M_3 muscarinic receptors. Data reproduced from reference (38).

to oppose β-adrenoceptors, activation of which facilitates bladder relaxation during urine storage. It can therefore be postulated that, during bladder voiding, M_3 receptors cause direct smooth muscle contraction whereas M_2 receptors reverse sympathetically mediated smooth muscle relaxation and these two effects synergise to cause more efficient discharge of urine.

Interestingly, this role of M_2 receptors appears to be enhanced during aging (26). Another intriguing finding, which requires confirmation, is that following pelvic denervation or spinal cord injury, M_2 receptors contribute to direct contractile responses in the urinary bladder (44). Overall, the data suggest that both M_2 and M_3 receptors may be functionally operative in the detrusor, although their relative contributions may be altered during aging and by disease.

Parasympathetic nerves innervating the urinary bladder are endowed with pre-junctional inhibitory and facilitatory muscarinic receptors that are differentially activated, depending upon the frequency of nerve stimulation (10, 39, 45-50). At low frequences the inhibitory receptors are preferentially activated, whereas the facilitatory mechanism predominates at high frequencies. The prejunctional facilitatory muscarinic receptor appears to be M_1 in the rat (10, 46), rabbit (39) and human (48) urinary bladder and operates through a phospholipase C-protein kinase C signal transduction pathway (49). The identity of the pre-junctional inhibitory muscarinic receptor is at present a subject of controversy. It has been classified as M_2 or M_4 in the rat (10, 46, 51) rabbit (39, 47) and guinea-pig (50) bladder.

It has been proposed that the pre-junctional facilitatory M_1 muscarinic receptor in the bladder modulates parasympathetic excitatory input to the bladder and serves as an amplification mechanism during voiding. Although these receptors can be shown to modulate acetylcholine release and contractile responses under *in vitro* conditions, evidence for their involvement *in vivo* is rather tenous. For example, pirenzepine, which possesses selectivity for M_1 receptors, does not potently inhibit volume-induced bladder contractions in the anesthetized rat (38). Nevertheless, one cannot dismiss a role of facilitatory M_1 receptors in the setting of pathological conditions.

It is surprising that the role of central muscarinic receptors in bladder function has seldom been investigated given the obligatory role of spinal and supra-spinal regulatory mechanisms in the operation of the micturition reflex. Indeed, a notion which is rarely contemplated is that the therapeutic efficacy of antimuscarinics may be due, in part, to blockade of central muscarinic receptor blockade. It is possible that future endeavors to elucidate the central muscarinic subtypes involved in lower urinary tract function may uncover novel targets for drug intervention.

Therapeutics

Oxybutynin (52), propantheline (53) and more recently, tolterodine (54), are marketed anti-muscarinics for treating the symptoms of urge incontinence. Oxybutynin displays some modest (~ 7-fold) M_3 over M_2 selectivity whereas both propantheline and tolterodine are non-selective muscarinic receptor antagonists. In clinical trials, tolterodine has been shown to produce a slightly lower incidence of dry mouth, compared to oxybutynin, although the mechanistic basis of this observation is unclear (55). However, at tolerated therapeutic doses (1 and 2 mg, b.i.d), tolterodine produces only modest improvement in symptoms. Among the new chemical entities that are being clinically evaluated for urge incontinence, darifenacin (56), temiverine (NS-21) (57), vamicamide (58), YM 46303 (59) are in the most advanced stage of development. Darifenacin, which displays selectivity for M_3 receptors, is expected to cause less tachycardia owing to its low affinity for M_2 receptors but is expected to cause dry mouth. Vamicamide, temiverine and YM-46303 are muscarinic antagonists which possess negligible or modest

selectivity for M_3 receptors and are in different stages of clinical development for urinary incontinence. The bladder-selective properties of vamicamide results from selective accumulation in this tissue. Temiverine differs from other compounds in that it is also a potent calcium-channel blocker. Finally, sustained-release oral formulations of oxybutynin (60) are being developed for urge incontinence. While these products will clearly have a dosing advantage (q.d. as opposed to t.i.d. with conventional oxybutynin), it is unclear whether they would possess a better safety profile.

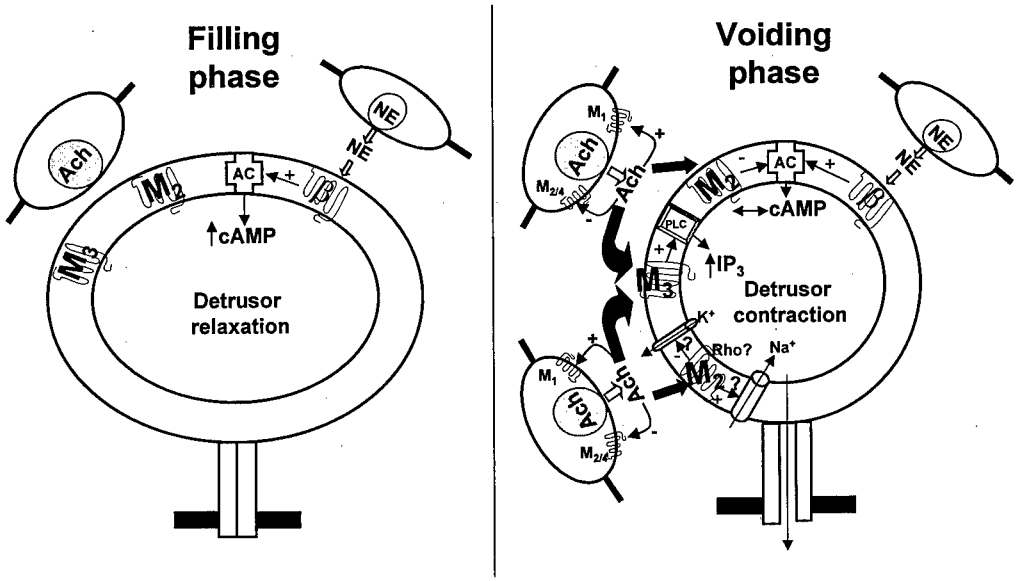

FIG. 4

A schematic diagram summarizing proposed mechanims underlying the stimulatory effects of parasympathetic nervous system on the voiding process. During the filling phase, the sympathoinhibitory drive to the bladder is tonically active and norepinephrine (NE) increases the compliance of the bladder through β-adrenoceptor mediated relaxation of the detrusor (via stmulation of adenylyl cyclase (AC)). The parasympathetic drive to the bladder, which is normally supressed during the filling phase, is augmented during the voiding phase. Acetylcholine (Ach), released from post-ganglionic cholinergic nerves interacts with post-junctional M_3 muscarinic receptors to cause direct detrusor contraction (via phospholipase C (PLC) stimulation) whereas activation of post-junctional M_2 muscarinic receptors inhibit β-adrenoceptor mediated stimulation of AC thereby causing reversal of the relaxant tone to the bladder. This dual mechanism causes more efficient and complete voiding of urine. Other postulated M_2 contractile mechanisms, demonstrated in other smooth muscles and which could also be operative in the bladder, include inhibition of K channels, activation of non-specific cation channels and stimulation of Rho proteins. The magnitude of the post-junctional response is also determined by the extent to which pre-junctional inhibitory (M_2/M_4) and facilitatory (M_1) muscarinic receptors modulate Ach release.

Conclusions

An extensive body of evidence, emanating from immunological, RT-PCR, northern blot and radioligand binding studies, suggests that the muscarinic receptor protein/mRNA population in the urinary bladder smooth muscle is comprised primarily of M_2/m_2 and M_3/m_3 receptor subtypes with the former predominating in number. The role of M_3 receptors in mediating direct detrusor contraction is undisputed (Fig. 4). Emerging evidence that M_2 muscarinic receptors cause smooth muscle contraction indirectly by inhibiting sympathetically (β-adrenoceptor)-mediated relaxation and may also contribute, in certain diseased states, to direct contractile responses (Fig. 4). Other contractile mechanisms involving M_2 muscarinic receptors, such as activation of a non-specific cationic channel and inactivation of K_{ATP} channels, may also perhaps be operative in the bladder and require further investigation (Fig. 4).

From a mechanistic standpoint, combined blockade of M_2 and M_3 muscarinic receptors would be therapeutically rewarding since this approach would evoke complete inhibition of cholinergically-evoked smooth muscle contractions. However, if either M_2 or M_3 receptors assumes a greater pathophysiological role in disease states, then selective antagonism of only one of the two receptors may be the more rationale approach. The ultimate therapeutic strategy is also influenced by which subtypes mediate the undesirable effects of anti-muscarinic blockade such as dry mouth. Additionally, since blockade of prejunctional (inhibitory and facilitatory) receptors can potentially influence the amount of acetylcholine released and, consequently, the magnitude of the post-junctional response, this element needs to be weighed in while targeting antagonists for one or more muscarinic receptors. Lastly, the effects of muscarinic receptor blockade in the central nervous system on bladder function, an issue which is seldom taken into consideration, should not be ignored.

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M_2 RECEPTORS IN GENITO-URINARY SMOOTH MUSCLE PATHOLOGY

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Summary

In vitro bladder contractions in response to cumulative carbachol doses were measured in the presence of selective muscarinic antagonists from rats which had their major pelvic ganglion bilaterally removed (denervation, DEN) or from rats in which the spinal cord was injured (SCI) via compression. DEN induced both hypertrophy (505±51 mg bladder weight) and a supersensitivity of the bladders to carbachol (EC_{50}=0.7±0.1 uM). Some of the SCI rats regained the ability to void spontaneously (SPV). The bladders of these animals weighed 184±17 mg, significantly less than the bladders of non voiding rats (NV, 644±92 mg). The potency of carbachol was greater in bladder strips from NV SCI animals (EC_{50}=0.54±0.1 uM) than either bladder strips from SPV SCI (EC_{50}=0.93±0.3 μM), DEN or control (EC_{50}=1.2±0.1 μM) animals. Antagonist affinities in control bladders for antagonism of carbachol induced contractions were consistent with M_3 mediated contractions. Antagonist affinities in DEN bladders for 4-diphenlacetoxy-N-methylpiperidine methiodide (4-DAMP, 8.5) and para fluoro hexahydrosilodifenidol (p-F-HHSiD, 6.6); were consistent with M_2 mediated contractions, although the methoctramine affinity (6.5) was consistent with M_3 mediated contractions. p-F-HHSiD inhibited carbachol induced contraction with an affinity consistent with M_2 receptors in bladders from NV SCI (pKb=6.4) animals and M_3 receptors in bladders from SPV SCI animals (pKb=7.9). Subtype selective immunoprecipitation of muscarinic receptors revealed an increase in total and an increase in M_2 receptor density with no change in M_3 receptor density in bladders from DEN and NV SCI animals compared to normal or sham operated controls. M_3 receptor density was lower in bladders from SPV SCI animals while the M_2 receptor density was not different from control. This increase in M_2 receptor density is consistent with the change in affinity of the antagonists for inhibition of carbachol induced contractions and may indicate that M_2 receptors or a combination of M_2 and M_3 receptors directly mediate smooth muscle contraction in bladders from DEN and NV SCI rats.

Key Words: muscarinic receptors, bladder, spinal cord injury, incontinence, denervation

Acetylcholine, through its action at muscarinic receptors on smooth muscle cells, is the primary neurotransmitter controlling bladder voiding (1, 2). Muscarinic receptor density and the contracile response of bladder smooth muscle to muscarinic stimulation are greatest in the dome and lowest in the base, allowing efficient bladder emptying (3). Pharmacological, biochemical and molecular data provide ample evidence that muscarinic receptors are heterogenous in nature (4, 5, 6).

Pharmacologic data, based on the actions of subtype selective antimuscarinic agents, can distinguish at least three distinct subtypes of muscarinic acetylcholine receptors (M_1, M_2, and M_3, 7). Molecular techniques have identified five muscarinic receptor subtypes (6) arising from five separate genes. Immunological and molecular studies revealed that most tissues including the urinary bladder express a mixture of subtypes (8, 9).

Binding and subtype selective immunoprecipitation studies demonstrate that the majority of muscarinic receptors in the urinary bladder are of the M_2 subtype (10, 11). On the other hand, pharmacological studies using subtype selective antagonists indicate that the M_3 receptor subtype mediates smooth muscle contraction (10). The M_2 receptor may be involved in inhibition of β-adrenergic receptor induced relaxation. A contribution of the M_2 receptor to contraction in normal bladder tissue can only be demonstrated indirectly, when the majority of M_3 receptors are inactivated in an environment of increased β-adrenergic receptor activation and when the tissue is prestimulated with a contractile agent such as potassium chloride (12, 13). The presence of prejunctional M_1 facilitory and M_2 inhibitory receptors on parasympathetic nerves innervating the rat bladder has been demonstrated both by acetylcholine release (14, 15) and muscle contraction studies (16).

Unlike other mammalian species, the normal rat bladder does not contain intramural ganglia (17). Bilateral ablation of the rat major pelvic ganglion (denervation, DEN) results in degeneration of bladder axons (18) and a rat unable to void. When the spinal cord of rats is damaged at T9 (SCI-decentralized), the rats frequently lose the ability to void spontaneously. The nonvoiding rat bladders hypertrophy. Consequently, both DEN and SCI results in hypertrophy of the urinary bladder. SCI rats have intact peripheral innervation of the bladder, although many can not void spontaneously apparently due to damage of the spinal cord which blocks the message to void from the micturition center. Thus, DEN and SCI are two different models of neurologic damage which can result in a nonvoiding animal. We compared the function and density of muscarinic receptor subtypes from the urinary bladders from SCI and DEN rats.

We measured the density of total, M_2 and M_3 receptors by subtype selective immunoprecipitation and calculated affinity values for a panel of muscarinic antagonists for inhibition of carbachol induced contractions three weeks after bladder denervation. Similar studies were performed on rats 10 days after SCI in order to compare the effects of DEN with decentralization on the density and function of muscarinic receptor subtypes in the rat urinary bladder.

Methods

Materials: Frozen normal rat bladders were purchased from Pel Freeze Biologicals (Rogers, AR). The following drugs or chemicals were obtained from the sources indicated: carbachol, sodium cholate, protease inhibitors, atropine (Sigma Chemical Company, St. Louis, Mo), methoctramine, 4-DAMP, 4-DAMP mustard, and p-F-HHSiD (Research Biochemicals International, Natick, MA), [^{3}H]QNB (43 Ci/mM Dupont-New England Nuclear Research Products, Wilmington, DE), pansorbin (Calbiochem Inc., La Jolla, CA), digitonin (Gallard-Schlesinger Industries Inc., Carle Place, NY).

Surgery: Rats (200-250 g female Sprague-Dawley rats from Ace Animals Inc., Boyertown, PA) were anesthetized with 2% isoflurane in oxygen and a midline incision was made in the lower abdomen. For bilateral denervation, both the left and right major pelvic ganglion were cauterized with a Valleylab Inc.(Boulder, CO) handstitching pencil attached to a Model SSE 2 solid state

electrosurgery device (Valleylab Inc., Boulder, CO). For sham operated animals, the plexus was exposed but left intact. For spinal cord injury a T8-T10 laminectomy was performed and the cord was compressed with a 35 g weight for 10 min at T9. After surgery, urine was expressed by the use of manual pressure on the lower abdomen of the animals twice daily.

Muscle Strips: Urinary bladders were removed from rats euthanized by decapitation. The urinary bladder body (tissue above the ureteral orifices) was dissected free of the serosa and surrounding fat. The bladder was divided in the mid-sagittal plane, then cut into longitudinal smooth muscle strips (approximately 4 mm x 10 mm). The muscle strips were then suspended with 1 g of isometric tension in tissue baths containing 15 ml of modified Tyrodes solution (125 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 23.8 mM $NaHCO_3$, and 5.6 mM glucose) and equilibrated with 95/5% O_2/CO_2 at 37 °C. The strips were tested for their ability to contract in response to electric field stimulation of 8 volts, 30 Hz, 1 ms duration. The electric field stimulation was generated by a solid-state square wave stimulator (Model S88, Grass Instruments, Quincy MA) interfaced through a stimulus power booster (Stimu-Splitter II, Med-Lab Instruments, Loveland, CO) in order to maintain the amplitude, duration, and shape of the stimulus signal which is transmitted to 12 tissue baths in parallel simultaneously. The 2.5 cm long serpentine shaped platinum electrodes are situated parallel to the long axis of the muscle strips approximately 1.25 cm apart in 15 ml organ baths (Radnoti Glass Technology, Monrovia, CA).

Carbachol Concentration-Effect: Following equilibration to the bath solution for 30 minutes, bladder strips were incubated for 30 minutes in the presence or absence of antagonist. Concentration-effect curves were derived from the peak tension developed following cumulative addition of carbachol (10 nM to 300 μM final bath concentration). An EC_{50} value for each strip was determined from an nonlinear least squares sigmoidal curve fit of the data (Origin, MicroCal Software, Inc., Northampton, Mass). The EC_{50} values determined in the presence of antagonist were used to generate Schild plots in order to calculate pA_2 values for each antagonist. If the slope of the Schild plot was not significantly different from unity, then the slope of the Schild plot was constrained to unity in order to calculate the pKb value. When the slope of the Schild plot was significantly less than unity, pA_2 values with slope are reported.

Immunoprecipitations: A two step solubilization procedure for immunoprecipitation (19) and antibodies (10) were used as previously described. Muscarinic receptor density is reported as fmoles/mg protein in the solubilized receptor preparations.

Statistical and data analysis: Results are reported as means ± S.E.M. The contractility data curves were generated by a curve fitting program (Origin, MicroCal Software, Inc., Northampton, Mass) based on a sigmoidal fit of the data for the concentration-effect curves and a linear fit for the Schild plots. Statistical analysis of multiple group comparison was performed by analysis of variance (ANOVA) with a post hoc Scheffé test (GB-STAT, Dynamic Microsystems, Silver Spring, MD) or Student's t test where appropriate. Statistically significant differences in the affinity values and departure from unity in the slopes derived from the Schild plots were determined using 95% confidence intervals.

Results

General Findings: DEN induced hypertrophy of the rat urinary bladder. DEN bladders weighed on average 505±51 mg (n=9) which was significantly more ($p<0.01$) than sham operated DEN bladders (98±5 mg, n=10). The bladders of SPV SCI animals weighed 184±17 mg (n=14), significantly less than the bladders of NV SCI rats (644±92 mg, n=10). Bladders from both SPV

and NV SCI animals weighed significantly more than bladders from sham spinal cord injured animals (105±5 mg, n=5). While the bladders from SCI animals contracted normally to electric field stimulation, DEN bladders did not contract (data not shown).

Agonist Affinity: There was no difference between the EC_{50} values of carbachol for inducing contractions in control and sham operated bladder strips (data not shown). As a consequence these values were pooled for comparison to DEN bladders. The EC_{50} of carbachol for inducing contractions in DEN bladders (0.71±0.09 μM) was significantly lower ($p<0.05$) than pooled sham operated and control bladders (1.26±0.21 μM). The EC_{50} of carbachol for inducing contractions in SPV SCI bladders (EC_{50}=0.93±0.3 μM) was not different from normal control or sham operated control SCI bladders (1.8±0.3 μM), however the EC_{50} of carbachol for inducing contractions in NV SCI bladders (EC_{50}=0.54±0.1 uM) was significantly lower than either control.

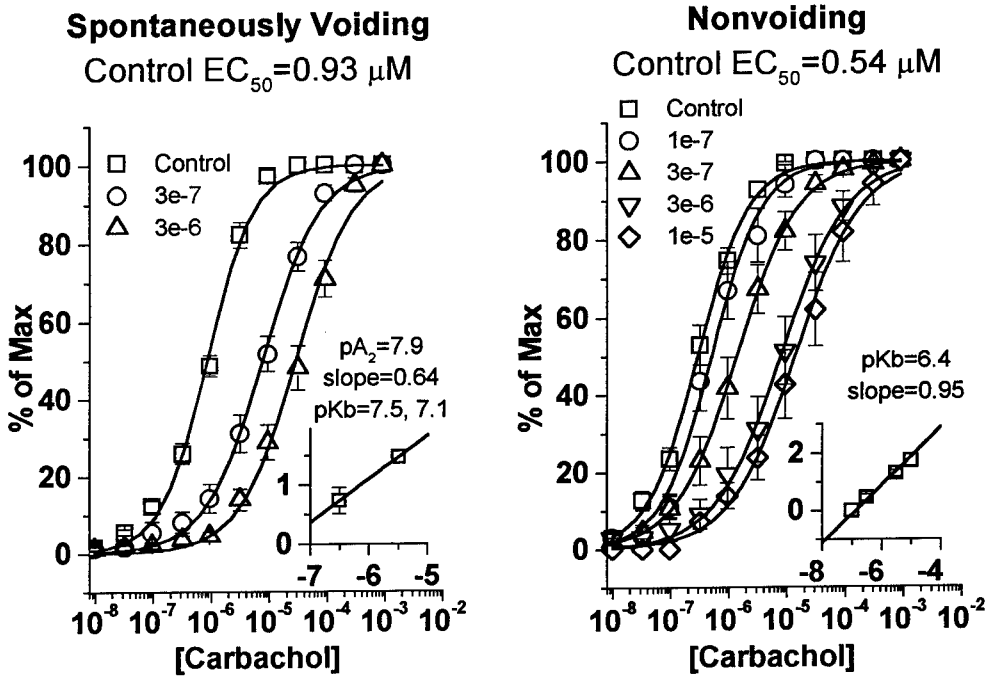

Fig. 1.
Carbachol Dose-Response Displacement curves and Schild plot (inserts) for p-F-HHSiD, Effect on Bladder Strips From Chronic Spinal Cord Injured Rats *in-vitro*. Each curve represents the average responses of muscle strip preparations expressed as the percent of each individual strip's maximal carbachol response. These maximal responses (average g ± S.E.M.) for SPV SCI bladders were 2.5±0.3 for control (n=15); 3.1±0.3 (n=14) and 2.2±0.2 (n=14) for 0.3 and 3 μM p-F-HHSiD respectively. The maximal responses for NV SCI bladder strips were 2.4± 0.3 for control (n=17); 2.7±0.3 (n=6), 2.1± 0.2 (n=11), 2.6± 0.5 (n=13) and 2.5± 0.6 (n=8) for 0.1, 0.3, 3, and 10 μM p-F-HHSiD respectively. There was no significant difference in maximum between groups.

Antagonist Affinities: Schild analysis of the shift in the carbachol dose response curves for a series

of muscarinic receptor antagonists revealed a dose dependent competitive inhibition of bladder muscle contraction. As previously shown using PZP, methoctramine, 4-DAMP, and p-F-HHSiD, muscarinic receptor antagonists inhibited carbachol stimulated muscle contractions in control bladders at a concentration consistent with M_3 receptors directly mediating muscle contraction (10, 16). However, as previously shown (20), in DEN bladders the affinities of 4-DAMP (pKb=8.5±0.2) and p-F-HHSiD (pKb=6.5±0.4) for inhibiting carbachol induced contractions are consistent with M_2 receptors directly mediating muscle contraction (7, 12). The affinity of methoctramine for inhibiting carbachol induced contractions in the DEN rat bladder (pA_2=6.5±0.5) is consistent with M_3 receptors directly mediating muscle contraction. This affinity is not different from the affinity of methoctramine in control bladders. However, the slope of the Schild plot for DEN bladders was 0.60, significantly less than unity, as opposed to a slope not different from unity in control bladders. In sham operated controls, the affinities of methoctramine (pKb=6.2±0.4) and p-F-HHSiD (pKb=7.7±0.6) for inhibiting carbachol induced contractions are consistent with M_3 receptors directly mediating muscle contraction as is the case in normal bladders. The affinity of p-F-HHSiD for inhibiting carbachol induced contractions in bladders from NV SCI rats is consistent with M_2 receptors directly mediating muscle contraction (pKb=6.4±0.2). However, in bladders from SPV SCI and sham operated SCI animals, the affinity of p-F-HHSiD (pA_2=7.9±0.2, slope=0.65, pKb=7.5±0.2, respectively) is consistent with M_3 receptors mediating this response or possibly M_2 and M_3 receptors in SPV SCI animals (Figures 1, 2).

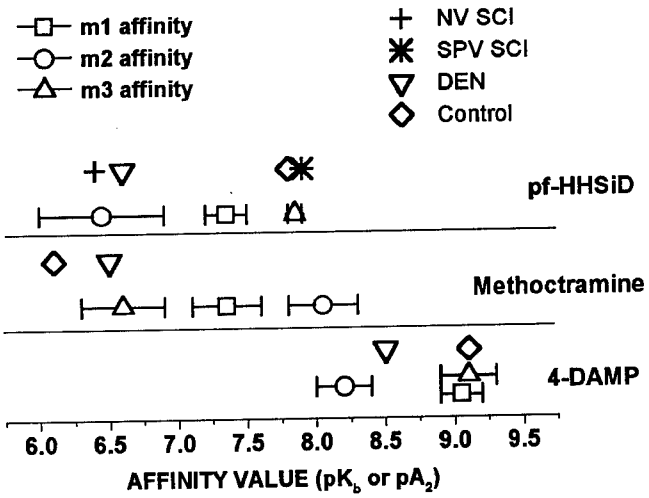

Fig. 2.

Antagonist Affinity for Inhibition of *in-vitro* Bladder Strip Contraction from Spinal Cord Injured and Denervated Rats. Affinities were determined as described in methods.

Immunoprecipitation: The total muscarinic receptor density (fmoles/mg solubilized protein) in DEN bladders was significantly ($p<0.01$) higher than in either sham operated or in unoperated control bladders. Also as can be seen in figure 3, the density of M_2 receptors was also significantly higher in denervated bladders than in either sham operated ($p<0.05$) or unoperated controls ($p<0.01$). There was no difference in the density of M_3 receptors. The sum of the M_2 and M_3 receptors precipitated accounted for 87%, 92%, and 87% of the total receptors solubilized for unoperated control, sham operated control, and denervated bladders, respectively.

Total muscarinic receptor density in bladders from NV SCI animals was significantly higher ($p<0.01$) than in control bladders. The increase in M_2 receptor density accounted for all this increase, with no change in M_3 receptor density. Bladders from SPV SCI animals showed no change in total receptor, or M_2 receptor density although there was a decrease in M_3 receptor density. The sum of the M_2 and M_3 receptors precipitated accounted for 83%, 89%, and 87% of the total receptors solubilized in bladders from NV SCI, SPV SCI, and control animals, respectively.

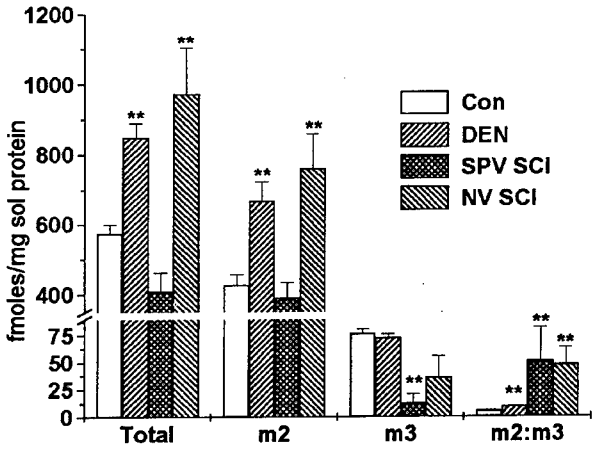

Fig. 3.

Precipitation of M_2 and M_3 Muscarinic Receptor Subtypes from the Bladder of Control, DEN, SPV SCI, and NV SCI Rats. Receptors were labeled with [^{3}H] QNB and solubilized as described in Luthin *et. al.* (19). Data shown are average fmoles of receptor/mg solubilized protein ± S.E.M. from individual DEN (n=4) and NV SCI (n=6) bladders, pooled normal (n=4) and pooled SPV SCI bladders (n=2) or the ratio of m2:m3 receptors. Protein concentration in the solubilized receptor preparation was approximately 8% of the protein concentration in the crude homogenate. As compared to filtration binding, approximately 50% of the muscarinic receptors were solubilized (data not shown). ** denotes significant difference ($p<0.01$) from control.

Discussion

Bilateral ablation of the major pelvic ganglion produced rats unable to void. The bladders of these animals were severely hypertrophied weighing 515% of control bladders. Compression injury to the spinal cord of rats at T9 yielded two groups of animals with respect to their ability to void. Some of the animals regained the ability to void spontaneously. The bladders from the SPV SCI animals were mildly hypertrophied (187% of control). The rats that regained the ability to void spontaneously on days 1 and 2 post-injury tended to have smaller bladders than the rats whose spontaneous voiding recovered later (data not shown). The bladders from NV SCI rat were severely hypertrophied weighing 657% of control bladders. Consistent with other reports, in bladders from both NV SCI and DEN rats, we observed a shift to the left in the carbachol

concentration effect curve termed "increased responsiveness" or "denervation induced supersensitivity" (21, 22). The EC_{50} of carbachol for inducing contractions in these bladders was significantly lower than in control bladders. The bladders from SPV SCI rats did not show a supersensitivity to carbachol.

Despite the predominance of M_2 subtypes in rat bladder, pharmacologic evidence based on the affinity of a panel of subtype selective muscarinic antagonists is most consistent with M_3 muscarinic receptors directly mediating smooth muscle contraction (10, 20). Based on the pharmacological data obtained with DEN and NV SCI rat bladders compared with normal and sham operated control rat bladders, it appears that in these bladders, M_2 receptors provide a contractile function which is mediated by M_3 receptors in normal and spontaneously voiding SCI bladders (Figure 2). The reason that both 4-DAMP and p-F-HHSiD produced affinities consistent with M_2 mediated contraction while methoctramine yielded an affinity consistent with M_3 mediated contraction in DEN bladders is unknown. The slope of the Schild plot of p-F-HHSiD for inhibition of carbachol induced contractions of SPV SCI bladders was less than one as was the slope of the Schild plot for methoctramine inhitition of bladder contraction from DEN rats. One explanation for a Schild plot having a slope of less than one is the interaction of more than one receptor subtype mediating the response. It is possible that a combination of M_2 and M_3 receptors is mediating contraction in the denervated and spinal cord injured hypertrophic bladder.

In general agreement with others, we show that denervation of the rat urinary bladder induced an increase in the density of total muscarinic receptors (22, 23). The apparent change in function of the M_2 receptor could be the direct result of the selective increase in M_2 receptor density in DEN and NV SCI bladders (Figure 3). No increase in M_2 receptor density was seen in bladders from SPV SCI rats. However, the M_3 receptor density in these bladders was lower than in normal bladders.

The M_2:M_3 receptor ratio is similar in both NV and SPV SCI bladders. Even though this ratio is greater in SPV SCI rat bladders than in DEN bladders, M_3 receptors mediate carbachol induced contractions in SPV SCI bladders while M_2 receptors mediate these contractions in DEN bladders. Data from selective M_3 receptor alkylation studies using 4-DAMP mustard show that even though a major proportion of M_3 receptors are inactivated, carbachol induced contractions are still mediated by M_3 receptors (24). Based on these finding, it appears that the absolute density of M_2 receptors, is more important than the M_2:M_3 ratio in determining which subtype mediates contraction. However, other differences resulting from adaptation of the bladder induced by either hypertrophy or the increased mechanical stretch imposed on the hypertrophied bladders may not allow for straightforward comparison of the results between these paradigms.

In conclusion, in both DEN and NV SCI bladders unlike SPV SCI bladders, there is an increase in M_2 muscarinic receptor density with no change in M_3 receptor density. The bladders from these animals are severely hypertrophied and show an increased responsiveness to carbachol. The affinity of muscarinic receptor antagonists for inhibition of carbachol induced smooth muscle contraction switches from being consistent with M_3 receptor mediated contraction in control bladders to M_2 mediated or a combination of M_2 and M_3 receptor mediated contraction in DEN and NV SCI bladders.

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M_2 SIGNALING IN SMOOTH MUSCLE CELLS

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Summary

M_2 receptor stimulation results in the gating of nonselective cation channels in several smooth muscle cell types. However the requirement for current activation includes a rise in cytosolic calcium mediated by M_3 receptor induced calcium release. This complex signaling system confers substantial complexity on the interpretation of pharmacological experiments. M_2 and M_3 receptor stimulation has also been linked to the inhibition of potassium channels in smooth muscle. These signaling events are likely to play important roles in excitation/contraction coupling.

Key Words: M_2 receptor, smooth muscle cells, M_2 signaling

Release of acetylcholine from parasympathetic nerves is the major determinant of tone in airway, gastrointestinal, and genitourinary smooth muscle. The signaling mechanisms linking M_3 muscarinic receptors to an increase in $[Ca^{2+}]_i$ in smooth muscle is well established, whereas the molecular pathways and functional importance of signaling by postsynaptic M_2 receptors on smooth muscle is poorly understood, despite the fact that these receptors comprise approximately 80% of the receptors in this tissue. In the heart, M_2 receptors couple to the opening of inward rectifier potassium channels through the direct binding of βγ G protein subunits to the channel (1-4). While this signaling pathway does not exist in smooth muscle cells, data from several laboratories indicate that M_2 receptors on smooth muscle cells are linked to the opening of another class of membrane ion channels, nonselective cation channels, and inhibit potassium channel gating. This brief report will summarize information about the coupling processes linking M_2 receptors and ion channels in smooth muscle.

M_2 Receptor Coupling to Nonselective Ion Channels in Smooth Muscle

The application of acetylcholine or muscarinic agonists to isolated smooth muscle cells results in a stereotypic electrical response consisting of the transient activation of calcium-dependent potassium and chloride channels, associated with the release of intracellular calcium, and the sustained activation of nonselective cation channels. There is substantial agreement from several laboratories that activation of nonselective cation channels which underlie slow excitatory postsynaptic potentials (EPSP's) observed following parasympathetic nerve stimulation of smooth muscle preparations (5), requires M_2 receptor stimulation. The evidence for M_2 receptor

involvement includes block of coupling by pertussis toxin (6-9), and the use of semi-selective receptor antagonists and G_i/G_o selective antibodies (9,10). However, the coupling is not as simple as a unitary pathway between a single muscarinic receptor subtype and channel gating. While a rise in $[Ca^{2+}]_i$ is not sufficient to activate the nonselective cation current (I_{Cat}) (11-13), the current is strongly facilitated by an increase in $[Ca^{2+}]_i$ (11,14), resulting in a signaling process in which a rise in $[Ca^{2+}]_i$, as well as M_2 receptor binding, is required for channel opening (9,10).

In physiological terms, the dual nature of the coupling pathway translates to a requirement for simultaneous stimulation of M_2 and M_3 receptors for current activation. This, of course, is exactly what occurs when acetylcholine molecules are released from presynaptic nerve terminals and bind the mixed population of M_2 and M_3 receptors expressed on the surface of smooth muscle cells. In the laboratory, however, the dual requirement of M_2 receptor stimulation and a rise in $[Ca^{2+}]_i$ confers substantial interpretive difficulty on physiological experiments attempting to assess the functional importance of M_2 receptors. Figures 1 and 2 illustrate this point and demonstrate the requirement of simultaneous M_2 receptor coupling and increased $[Ca^{2+}]_i$ to activate I_{Cat}; the experiments shown are from single equine tracheal myocytes loaded with the calcium-sensitive fluorophor, fura-2 (9,10). As shown in Figure 1, if calcium release is evoked (and subsequent release prevented) by exposure to caffeine, stimulation of M_2 and M_3 receptors is not sufficient to activate I_{Cat}. The receptor subtype requirement is further dissected in Figure 2.

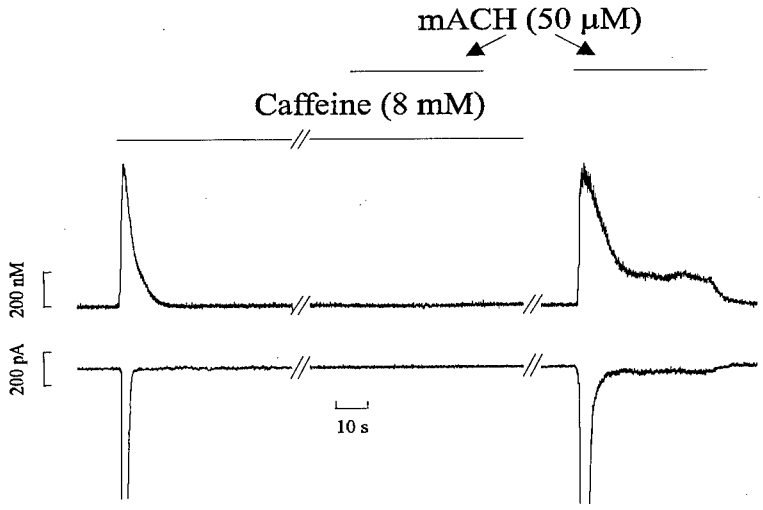

Fig. 1

Muscarinic stimulation, but not calcium release, activates an I_{Cat} current. Application of caffeine to a single smooth muscle cell voltage-clamped at –60 mV induces a transient increase in $[Ca^{2+}]_I$ (upper trace) and a transient calcium-activated chloride current (lower trace). Application of methacholine (mACH) to the same cell following calcium refilling activates the transient responses, but also a sustained rise in $[Ca^{2+}]_I$ and a sustained, noisy I_{Cat} current. Used with permission from Reference 9.

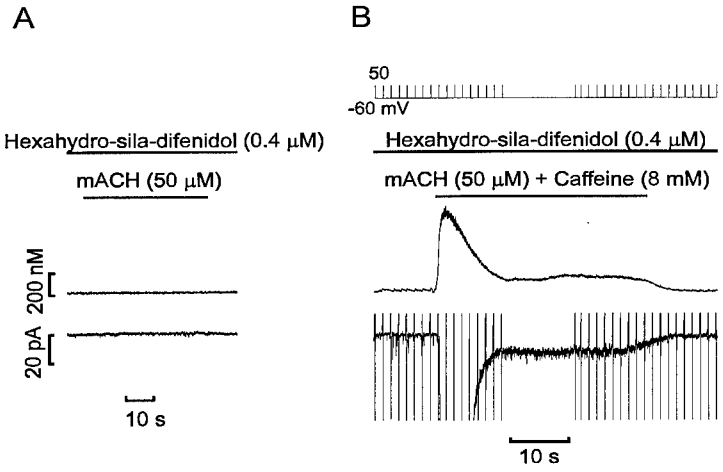

Fig. 2

Muscarinic activation of I_{Cat} requires M_2 receptor binding and an increase in $[Ca^{2+}]_i$. A. The M_3 receptor antagonist hexahydro-sila-difenidol blocks muscarinic release of Ca^{2+} (and the attendant calcium-activated chloride current) and I_{Cat}, indicating the M_2 receptor stimulation alone is not sufficient to activated I_{Cat}. B. Simultaneous application of methacholine to stimulate free M_2 receptors, and release of Ca^{2+} with caffeine, activates I_{Cat}. Used with permission from Reference 9.

In the presence of a concentration of the semi-selective M_3 receptor antagonist hexahydro-sila-difenidol, predicted to block 97% of M_3 receptors and 28% of M_2 receptors, methacholine does not stimulate calcium release and I_{Cat} is not activated. Thus, stimulation of free M_2 receptors alone is not sufficient to activate the current. Similarly, in the presence of 0.2 μM methoctramine (predicted to block 95% of M_2 and 15% of M_3 receptors), calcium release and activation of the calcium-activated chloride current occur normally, but I_{Cat} channels do not open (not shown). However, activation of the current can be reconstituted by separately inducing calcium release with caffeine at the same time as M_2 receptor stimulation. The consequence for organ physiology studies is apparent from Figure 2A, which shows that functional block of an M_2 receptor signaling process is achieved with M_3 receptor antagonism.

M_2 Mediated Inhibition Of Potassium Currents In Smooth Muscle

Muscarinic receptor stimulation results in a profound inhibition of potassium conductance in smooth muscle. Calcium-activated potassium channels are strongly inhibited both by release of intracellular calcium, thus eliminating the calcium release events that drive spontaneous channel activity (15,16), and by a membrane delimited inhibitory pathway between M_2 receptors and single K_{Ca} channels (17-21). Figure 3 illustrates these dual inhibitory coupling mechanisms. A single, voltage-clamped smooth muscle cell is depolarized to activate potassium channels;

spontaneous transient outward currents (STOCs) are seen superimposed on the whole-cell current. These currents are strongly suppressed following the stimulation of calcium release with caffeine. Following this suppression of potassium conductance associated with calcium release, application of methacholine further suppresses the outward potassium current. This calcium-independent effect is thought to be particularly important because cholinergic stimulation of smooth muscle results in an increase in $[Ca^{2+}]_i$ as well as a sustained depolarization, two events that would otherwise strongly increase calcium-activated potassium channel activity.

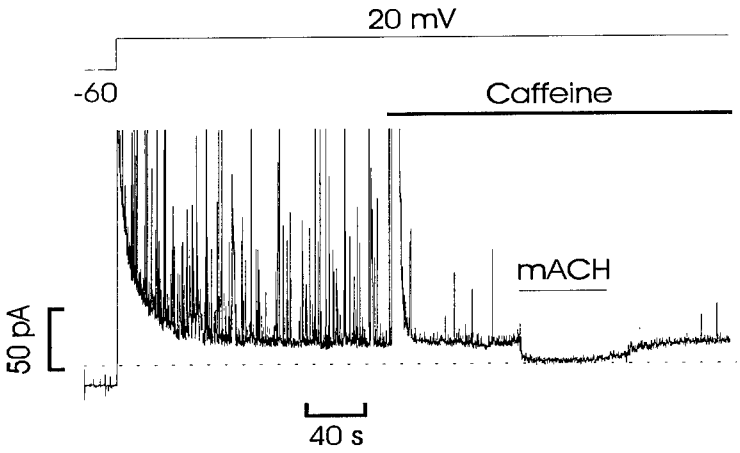

Fig. 3

Methacholine inhibits STOCs and whole-cell K_{Ca} current in the presence of caffeine. STOC activity and K^+ currents were evoked by step depolarization to 20 mV. After inactivation of much of the voltage-dependent current, application of caffeine induced an initial burst of K_{Ca} current (partially cut-off) and inhibited STOC activity. In the continued presence of caffeine, preventing subsequent calcium release, methacholine abolished the remaining STOCs and decreased the sustained K^+ current. From Reference 21.

The inhibition of K_{Ca} channel activity at a fixed level of calcium is likely to be an important component of the sustained postsynaptic excitatory potential.

Postreceptor Coupling Mechanisms

Neither the processes interposed between M_2 receptor binding and I_{Cat} activation, nor the molecular identity of the I_{Cat} channels themselves, have been determined. Although antibodies directed against the C-terminal regions of inhibitory G protein α subunits block activation of I_{Cat} (9), these experiments do not discriminate between $G\alpha$ or $G\beta\gamma$ activation of the channel or related regulatory proteins. M_2 receptors in atrial cells directly couple to the GIRK family of inward rectifier potassium channels through the binding of $\beta\gamma$ subunits to the channel (1,3). Similarly,

Ca^{2+} channels at presynaptic nerve terminals are inhibited by G-protein–coupled receptors acting through a membrane delimited pathway (22-24). The binding site for βγ interaction with target proteins has recently been localized using a peptide fragment of adenylyl cyclase 2 (25). This fragment blocks $G_{\beta\gamma}$ stimulation of adenyl cyclase 2, phospholipase-β3, β-ARK, GIRK1 potassium channels (25), and phosphoinositol 3 kinase γ (26), and inhibits neuronal Ca^{2+} channels (3,27-29). A common sequence has been identified in these target proteins consisting of a QXXER motif, or slight variant thereof. Interestingly, smooth muscle cells, in which M_2 receptor expression is abundant, express neither GIRK1, nor α_{1A}/α_{1B}, ion channels that have been associated with M_2 receptor signaling through $G_{\beta\gamma}$. Rather, the α_{1C} subunit of voltage-dependent calcium channels expressed in smooth muscle cells does not contain the QXXER domain, explaining the absence of G protein mediated inhibition of Ca^{2+} currents in smooth muscle.

Conclusions

Substantial evidence indicates the M_2 receptor stimulation is coupled to alterations in ion channel gating in smooth muscle cells. The molecular details underlying this connection, and in some cases the structure of the target channel, have yet to be determined. It is expected that reconstitution of these signaling pathways in heterologous expression systems will shed considerable light on these processes, as well as provide information that will allow specific hypotheses about the functional importance of these coupling processes to be critically examined

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ROLE OF M_2 MUSCARINIC RECEPTORS IN AIRWAY SMOOTH MUSCLE CONTRACTION

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Summary

Airway smooth muscle expresses both M_2 and M_3 muscarinic receptors with the majority of the receptors of the M_2 subtype. Activation of M_3 receptors, which couple to G_q, initiates contraction of airway smooth muscle while activation of M_2 receptors, which couple to G_i, inhibits β-adrenergic mediated relaxation. Increased sensitivity to intracellular Ca^{2+} is an important mechanism for agonist-induced contraction of airway smooth muscle but the signal transduction pathways involved are uncertain. We studied Ca^{2+} sensitization by acetylcholine (ACh) and endothelin- 1 (ET-1) in porcine tracheal smooth muscle by measuring contractions at constant $[Ca^{2+}]$ in strips permeabilized with Staphylococcal α-toxin. Both ACh and ET-1 contracted airway smooth muscle at constant $[Ca^{2+}]$. Pretreatment with pertussis toxin for 18-20 hours reduced ACh contractions, but had no effect on those of ET-1 or GTPγS. We conclude that the M_2 muscarinic receptor contributes to airway smooth muscle contraction at constant $[Ca^{2+}]$ via the heterotrimeric G-protein G_i.

Key Words: acetylcholine, airway smooth muscle contraction, endothelin, G-protein G_q, G-protein G_i, staphylococcal α-toxin

Airway smooth muscle expresses endothelin (ET) and both M_2 and M_3 muscarinic receptors with greater than 80% being of the M_2 subtype (1-5). Muscarinic M_3 and ET receptors couple to phospholipase C to produce increases in inositol triphosphate (IP_3) and diacylglycerol (DAG) via the heterotrimeric G-protein G_q. Activation of the M_2 muscarinic receptor inhibits adenylyl cyclase via interaction with members of the pertussis toxin-sensitive G-protein family G_i. Activation of receptors which couple to G_q initiates contraction of airway smooth muscle while activation of receptors which couple to G_i inhibits β-adrenergic receptor-mediated relaxation.

A rise in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) has long been recognized as the normal trigger for smooth muscle contraction. However, it is now widely appreciated that many contractile agonists act both to increase $[Ca^{2+}]_i$ and to enhance the effectiveness of Ca^{2+} for inducing contraction (6-12). The latter phenomenon can be readily demonstrated in membrane-permeabilized smooth muscle strips as a leftward shift of the force-$[Ca^{2+}]$ curve or as an agonist-induced contraction at constant $[Ca^{2+}]$. The signal transduction pathways involved in the

regulation of Ca^{2+} sensitivity are complex and smooth muscle type-specific. In most smooth muscle preparations, agonist-induced enhancement of Ca^{2+} sensitivity or potentiation of Ca^{2+}-induced contractions involves a G-protein mediated cascade that results in inhibition of myosin light chain phosphatase (9,13,14). This leads to an increase in the level of myosin light chain phosphorylation with increased numbers of attached cross bridges and increased force. Tyrosine kinases, protein kinase C, and thin filaments such as calponin are also involved (15-17).

A role for M_2 muscarinic receptors in the mediation of airway smooth muscle contraction has not been demonstrated. Since M_2 muscarinic receptors, but not M_3 receptors, couple to G_i, the contribution of M_2 receptors to acetylcholine-induced contraction of airway smooth muscle can be inferred from studies of the role of G_i in contraction. Hence, we examined the effects of inhibition of G_i with pertussis toxin on cholinergic contractions of permeabilized porcine tracheal smooth muscle strips at constant $[Ca^{2+}]$.

Methods

Tissue Preparation. Young adult swine (50-70 lbs) were sedated with ketamine (40 mg/kg i.m.), anesthetized with sodium pentobarbital (30 mg/kg i.v.), and killed by exsanguination through the femoral arteries. The tracheae were removed and placed in cold physiological salts solution for transport to the laboratory. Tissues were stored for up to 48 hrs at 4°C in similar solutions bubbled with 95% O_2/5% CO_2. Pairs of tracheal smooth muscle strips 0.2-0.3 mm in width and ~12 mm in length were dissected from the posterior aspect of the upper half of the trachea using a binocular microscope and were tied at either end with 6-0 silk suture for later attachment to a chamber and force transducer (Grass FT-03).

Pertussis Toxin Treatment. Prior to α-toxin permeabilization, smooth muscle strips were incubated in the presence or absence of pertussis toxin (20 μg/ml × 18-20 hrs at 31°C) in 500 μl of medium in a humidified atmosphere of 5% CO_2 in air. Preliminary results indicated better preservation of contractile responses in tissues pretreated at this reduced temperature. The culture medium was Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 240 ng/ml amphotericin B, and 100 U/ml nystatin. Pertussis toxin was prepared as a 500 μg/ml stock solution in water.

Permeabilization With Staphylococcal α-toxin. Before mounting in the chambers all tissue strips were incubated for 50-60 min with 830 U/ml α-toxin in 300 μl of relaxing solution ($[Ca^{2+}] = 10^{-9}$ M, composition given below).

Experimental Solutions. Pairs of permeabilized smooth muscle strips were mounted vertically and studied simultaneously in identical 800 μl chambers made from 1/4 dram glass shell vials. The chamber solution was stirred continuously with a magnetic bar. $[Ca^{2+}]$ was changed by flushing the chambers with ~4ml of solution added through polyethylene tubing to the bottom of the chamber. Excess solution was removed by suction at the top. All solutions contained (in mM) 130 K propionate, 20 mono[tris(hydroxymethyl)-aminomethane]maleate, 7.0 $MgCl_2$, 4.6 Na_2ATP, 2.0 ethyleneglycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 2.0 phosphocreatine, and 1.0 dithiothreitol plus 8 U/ml creatine phosphokinase, 10^{-6} M leupeptin, and sufficient $CaCl_2$ to provide the desired concentration of free Ca^{2+} assuming a Ca^{2+}-EGTA dissociation constant of 3×10^{-7} M. Solutions of 10^{-9} M and 3×10^{-4} M free Ca^{2+} were prepared and carefully adjusted to pH 7.10. Solutions of intermediate $[Ca^{2+}]$ were then made by mixing these solutions in appropriate proportions. All experiments were performed at room temperature.

Experimental Protocols. Effects of contractile agonists were evaluated in pairs of smooth muscle strips that were preincubated, permeabilized, and studied in parallel by identical methods, except for the presence or absence of pertussis toxin during pretreatment. Preliminary experiments indicated that baseline forces of ~2 mN were optimal for active force generation in these tissues. Hence, after permeabilization the tissues were bathed with relaxing solution (10^{-9} M free Ca^2) and stretched until a stable baseline force of ~2 mN was obtained. All solutions used here and subsequently contained 10^{-6} M calcium ionophore A23187 to deplete intracellular Ca^{2+} stores. After 20 min the $[Ca^{2+}]$ was increased to 10^{-7} M, a value which typically increased force by ~5% of the maximum Ca^{2+} response. Cumulative additions of GTP, acetylcholine (ACh), atropine, endothelin-1 (ET-1), and GTPγS were then made at 10-15 min intervals. All drugs were then washed out with 3×10^{-4} M free Ca^{2+} solution and the difference between the maximum force obtained in this solution and the force obtained in the relaxing solution prior to the protocol was defined as the maximum Ca^{2+}-induced force in that smooth muscle strip.

Data Analysis. Drug-induced changes in isometric force at constant $[Ca^{2+}]$ were normalized by the change in force induced by high $[Ca^{2+}]$ in that strip. The GTPγS response was measured relative to the value obtained prior to addition of GTP. Effects of pertussis toxin pretreatment on force responses were analyzed by paired two-tailed t-tests with n = number of animals and $P < 0.05$ considered significant.

Drugs and Chemicals. Culture reagents were purchased from Life Technologies (Gaithersburg, MD) and α-toxin was obtained from Calbiochem (La Jolla, CA). All other drugs and chemicals were from Sigma (St. Louis, MO).

Results

Figure 1 shows a typical protocol in which 10^{-5} M ACh induced contraction of an α-toxin-permeabilized strip at constant 10^{-7} M free Ca^{2+} in the presence of 5×10^{-6} M GTP. Atropine reversed the ACh response. Subsequent additions of 10^{-7} M ET-1 and 10^{-4} M GTPγS also produced contractions.

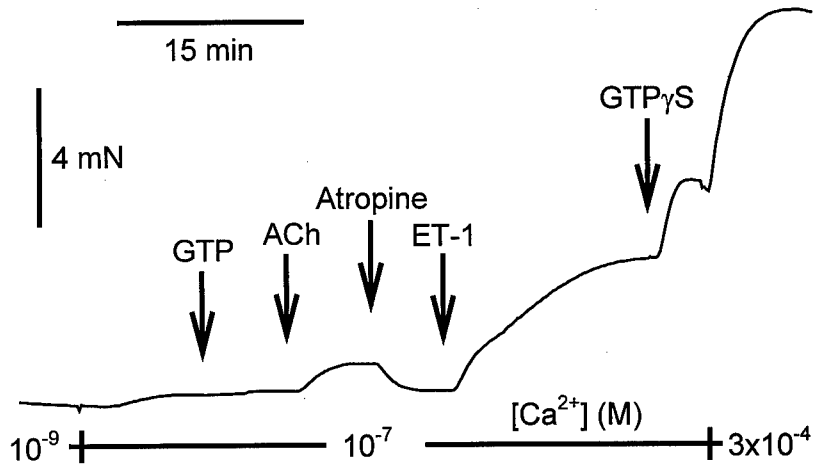

Fig. 1

Force tracing from a representative pig tracheal smooth muscle strip permeabilized with α-toxin and contracted at constant $[Ca^{2+}]$ by ACh, ET-1, and GTPγS.

To investigate the role of the heterotrimeric G-protein, G_i , in the potentiation of Ca^{2+}-induced contractions by ET-1 and ACh, we studied strips pretreated with pertussis toxin to inactivate G_i. Strips were permeabilized with α-toxin and studied as shown in Figure 1. Contractions to ACh were diminished in pertussis toxin-treated strips while responses to ET-1 and to GTPγS were unaffected (Figure 2). A summary of data from 11 experiments with smooth muscle strips from 9 animals is given in Figure 3. A 28% decrease in ACh-induced force in pertussis toxin-treated strips was statistically significant (P = 0.027, n = 9) while responses to other stimuli did not differ between treated and control tissues. Maximum Ca^{2+}-induced increases in force also did not differ between treated and control strips (6.02 ± 1.02 vs. 6.37 ± 1.16 mN, P = 0.55, n = 9).

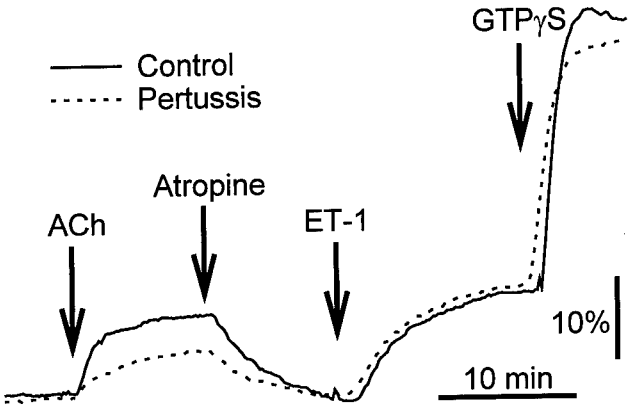

Fig 2.

Force (as % of Ca^{2+}-induced) recorded at constant [Ca^{2+}] in a representative pair of permeabilized smooth muscle strips treated and untreated with pertussis toxin.

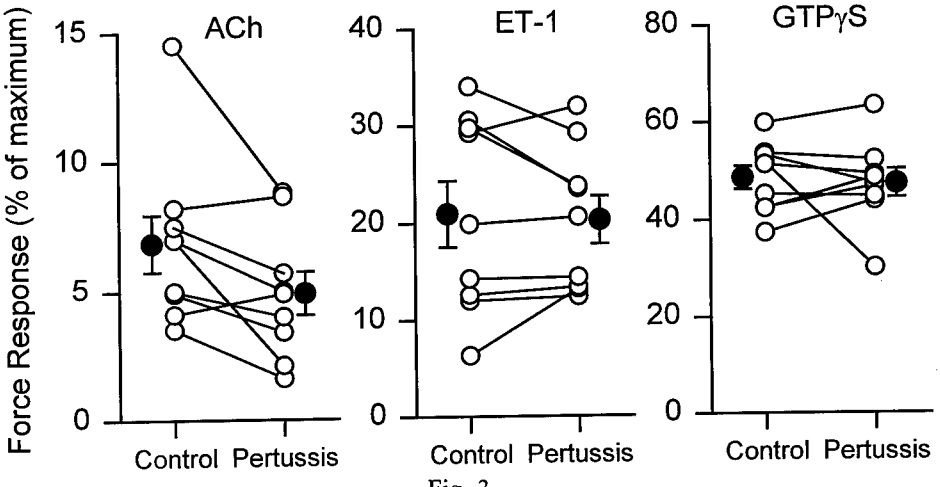

Fig. 3.

Active force responses to ACh, ET-1, and GTPγS in permeabilized tracheal smooth muscle strips. o denotes individual animals. ǂ denotes mean±SE, n = 9. Figure is modified from ref. 22.

Discussion

Muscarinic cholinergic receptors of types M_3 and M_2 are known to couple to the heterotrimeric (large) G-proteins G_q and G_i, respectively, in airway smooth muscle (18). ET_A and ET_B receptors are expressed in porcine airway smooth muscle (5) and each of these receptor subtypes is thought to couple to G_q as well as to G_i (19). G_q activation initiates contraction of airway smooth muscle through release of Ca^{2+} from intracellular stores and enhancement of Ca^{2+} entry, while G_i activation antagonizes relaxation through inhibition of adenylyl cyclase (2,20,21). Although agonist stimulation is known to also increase contractile sensitivity to Ca^{2+}, the roles of G_q and G_i in the enhancement of Ca^{2+}-sensitivity are not known.

Permeabilized smooth muscle preparations have been used extensively to characterize the cellular mechanisms that regulate Ca^{2+} sensitivity. Although the chemicals used to permeabilize the cell membrane may alter the function of relevant cellular pathways, Staphylococcal α-toxin is thought to be less injurious to tissues than detergents such as β-escin. In these studies we observed only minimal baseline drift or decay of contractions, consistent with good preservation of contractile mechanisms.

We tested the role of G_i in Ca^{2+} sensitization by ACh and ET-1 (22) by studying tissues incubated overnight in the absence or presence of pertussis toxin which is known to ADP-ribosylate and irreversibly inactivate G_i (23). Tracheal smooth muscle strips pretreated with pertussis toxin showed smaller ACh-induced contractions at constant $[Ca^{2+}]$ while the responses to ET-1 and GTPγS were unaltered (Figs. 2 and 3). These data indicate a novel function for G_i in mediating increases in Ca^{2+} sensitivity and hence, indicate that G_i and the M_2 muscarinic receptor contribute directly to contraction. This conclusion contradicts a generally held belief that the M_2 receptor/G_i pathway contributes to contraction only indirectly via inhibition of cAMP-mediated relaxation (20). In fact, our results agree with studies of the M_2-selective antagonist methoctramine in guinea pig airways which yielded Schild plots with slopes significantly less than unity (24), consistent with contraction through a heterogeneous population of receptors. The lack of effect of pertussis toxin on ET-1 responses indicates that endothelin couples primarily to G_q in porcine tracheal smooth muscle, consistent with other data from this laboratory showing stimulation of IP_3 production by ET-1, but no inhibition of GTP-stimulated adenylyl cyclase activity (25).

In summary, the signal transduction mechanisms of airway smooth muscle which enhance myofilament Ca^{2+} sensitivity involve both large and small G-proteins as has been demonstrated in other smooth muscle types. Studies with pertussis toxin indicate that G_i, in addition to its known ability to inhibit relaxation, enhances airway smooth muscle contraction directly by increasing Ca^{2+} sensitivity. In this tissue the effects of ET-1 are mediated primarily by a G protein other than G_i (presumably G_q), while the contractile effects of ACh are mediated, at least in part, by G_i. Hence, the M_3 receptor/G_q pathway and the M_2 receptor/G_i pathway appear to operate in parallel to produce contraction of airway smooth muscle. These multiple actions of ACh may contribute to the durability of cholinergic contractions in airway smooth muscle.

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EFFECTS OF INFLAMMATORY CELLS ON NEURONAL M_2 MUSCARINIC RECEPTOR FUNCTION IN THE LUNG

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Summary

In the lungs, acetylcholine released from the parasympathetic nerves stimulates M_3 muscarinic receptors on airway smooth muscle inducing contraction and bronchoconstriction. The amount of acetylcholine released from these nerves is limited locally by neuronal M_2 muscarinic receptors. These neuronal receptors are dysfunctional in asthma and in animal models of asthma. Decreased M_2 muscarinic receptor function results in increased release of acetylcholine and in airway hyperreactivity. Inflammation has long been associated with hyperreactivity and the role of inflammatory cells in loss of neuronal M_2 receptor function has been examined. There are several different mechanisms for loss of neuronal M_2 receptor function. These include blockade by endogenous antagonists such as eosinophil major basic protein, decreased expression of M_2 receptors following infection with viruses or exposure to pro inflammatory cytokines such as gamma interferon. Finally, the affinity of acetylcholine for these receptors can be decreased by exposure to neuraminidase.

Key Words: eosinophils, asthma, hyperreactivity, interferon-γ

It has been well documented that asthma is associated with both airway hyperresponsiveness and airway inflammation (1). Conditions associated with exacerbations of asthma often increase both airway responsiveness and the intensity of airway inflammation. Conversely, a clinical response to drugs that suppress inflammation, such as steroids, may be associated with a decrease in responsiveness (2). The finding of temporary hyperresponsiveness in non-asthmatics in whom airway inflammation has been induced either by viral infection, inhalation of allergen, or exposure to ozone further highlights the association of airway hyperresponsiveness with airway inflammation (3-5).

However, the mechanisms by which inflammatory cells cause hyperresponsiveness are unknown. Recently is has become clear that the increase in airway responsiveness that occurs after viral infections, allergen inhalation, or exposure to ozone may be mediated, at least in part, by increased vagally-mediated reflex bronchoconstriction (6). This is the case both in experimental animals and in humans.

The predominant neural control of the airways is provided by the parasympathetic nervous system (7-9). A role for the parasympathetic nerves is suggested since anticholinergic drugs such as atropine or ipratropium will, if given in adequate doses, cause bronchodilation in allergic asthma (6,10-12). Release of acetylcholine from the parasympathetic nerves is limited locally by inhibitory M_2 muscarinic receptors on the nerves. Since hyperresponsiveness is thus associated with increased release of acetylcholine from the parasympathetic nerves and with inflammation the role of inflammatory cells and inflammatory mediators on M_2 receptor function and expression was examined.

Inhibitory M_2 receptors and release of acetylcholine in the lungs

Release of acetylcholine from parasympathetic nerve endings in the airways is limited by negative feedback onto inhibitory M_2 muscarinic receptors on the nerve endings. The importance of these receptors is demonstrated by the fact that vagally-mediated bronchoconstriction can be potentiated by as much as 5-8 fold when these receptors are blocked with selective antagonists such as gallamine and methoctramine. Conversely, stimulating these receptors with the agonist pilocarpine decreases vagally-mediated bronchoconstriction by as much as 80% (13).

These inhibitory M_2 muscarinic receptors were originally demonstrated on the parasympathetic nerve endings in the airways of guinea pigs (13) and have subsequently been demonstrated in all species studied thus far , including humans (14-16). Neuronal M_2 muscarinic receptors are dysfunctional in three different guinea pig models of asthma; virus infection (17), antigen challenge of sensitized animals (18) and ozone exposure (19). There is also evidence that the neuronal M_2 receptors may be dysfunctional in some (20), but not all (21), patients with asthma.

The role of M_2 receptor dysfunction in airway hyperresponsiveness

In cases where the M_2 receptors are either permanently or transiently dysfunctional, loss of the negative feedback normally provided by these receptors would be expected to potentiate vagally-mediated reflex bronchoconstriction. Indeed, many agonists traditionally used to evoke bronchoconstriction for the purpose of determining airway responsiveness cause smooth muscle contraction, at least in part, by triggering a vagally-mediated reflex. Among these are histamine, cold air and allergen (22,23),. Furthermore, even methacholine, which is often assumed to work only via direct effects on airway smooth muscle, can cause a vagally-mediated reflex (24,25). Thus, there is the potential for M_2 receptor dysfunction, by increasing vagal acetylcholine release, to increase the airway response to inhaled agonists.

The contribution of M_2 receptor dysfunction to airway hyperresponsiveness has been demonstrated in experimental animals. In these models M_2 dysfunction can be prevented by a variety of pre treatments, generally directed either at preventing airway inflammation or interrupting the effects of specific inflammatory mediators (26-30). Such treatments not only prevent M_2 receptor dysfunction, but also prevent airway hyperresponsiveness.

Evidence that inflammatory cells are responsible for M_2 receptor dysfunction

The association of conditions characterized by airway inflammation with M_2 receptor dysfunction provides circumstantial evidence of a role for inflammatory cells in causing the M_2 receptor dysfunction. Thus studies were designed to establish a causative role for inflammation in loss of M_2 receptor dysfunction, and to determine the specific inflammatory mediators responsible.

Viral infections of the airways causes an intense inflammatory response characterized primarily by neutrophils and mononuclear cells. At the same time, M_2 receptor function is lost and vagally-mediated hyperresponsiveness occurs. Pretreating guinea pigs with cyclophosphamide before infecting them with parainfluenza virus depletes them of inflammatory cells and markedly attenuates the airway inflammatory response (27). Under these circumstances, M_2 receptor function is preserved in some, but not all, animals. When viral content of the lungs was determined in these experiments, it was demonstrated that animals with very high viral contents lost M_2 receptor function despite cyclophosphamide treatment, while in animals with lower viral contents, prevention of airway inflammation preserves M_2 receptor function. Thus viral infections cause M_2 receptor dysfunction via multiple mechanisms, some of which are leukocyte-dependent.

Inhalation of ozone also causes an influx of neutrophils into the lungs. When guinea pigs are depleted of neutrophils by pretreatment with cyclophosphamide, M_2 receptor function is preserved, and hyperresponsiveness prevented (26). Thus ozone-induced M_2 receptor dysfunction is wholly leukocyte-dependent.

In contrast to viral infections and ozone exposure, inhalation of allergens causes an inflammatory response composed predominantly of eosinophils. Prevention of this eosinophil influx can be achieved by pretreating animals with either antibody to interleukin-5 or with antibody to the adhesion molecule very late activation antigen-4 (VLA-4) (28,29). After either pretreatment, prevention of airway eosinophilia is accompanied by preservation of M_2 receptor function and inhibition of hyperresponsiveness.

Mechanisms of leukocyte-dependent M_2 receptor dysfunction

Eosinophils in allergen-induced M_2 receptor dysfunction: The mechanism by which leukocytes cause M_2 receptor dysfunction has been most clearly elucidated in the antigen-challenged guinea pig. As noted above, inhalation of antigen to which the animal has been previously sensitized causes an influx of eosinophils which appear to be a sine qua non for M_2 receptor dysfunction and airway hyperresponsiveness. Eosinophils are recruited not only to the lungs and airways, but appear to be specifically concentrated in and around the airway nerves (31). Eosinophils are present around and within the nerve bundles, around parasympathetic ganglia and along postganglionic nerve fibers. Furthermore, the degree of M_2 receptor dysfunction correlates with the number of eosinophils in contact with the airway nerves (31). This association of eosinophils with the airway nerves is also seen in airway tissue from patients who have died of severe asthma (31).

The association of eosinophils with the airway nerves further supports their role in M_2 receptor dysfunction. The recognition that many selective M_2 receptor antagonists are cationic (32) led to speculation that one of the strongly cationic proteins of the eosinophil granule might be acting as an endogenous antagonist. This speculation was strengthened when it was demonstrated that the most plentiful protein of the eosinophil, eosinophil major basic protein (MBP), is an allosteric antagonist at M_2 muscarinic receptors (33,34). The Ki for this interaction is 1.5×10^{-5}M, which is likely to be in the range of concentrations of this protein seen in sites of allergic response. Furthermore, in the range of concentrations tested, MBP had no effect on the M_3 muscarinic receptors, which mediate the contractile effect of acetylcholine on airway smooth muscle.

It was possible to determine whether major basic protein was blocking the M_2 receptor in vivo by taking advantage of the fact that poly-anionic substance such as heparin can bind and neutralize MBP. In addition, heparin can reverse the effect of MBP on binding of 3H-N-methyl scopolamine to M_2 receptors in vitro. When heparin was given intravenously to guinea pigs that had previously been challenged with allergen via inhalation, the heightened response to vagal stimulation was decreased by 50% within 20 minutes (35). This attenuation of vagal hyperresponsiveness was accompanied by return of full function of the M_2 receptor. Similar effects were seen when MBP was neutralized by treatment with other anionic substances such as poly-L-glutamic acid and partially desulfated, non-anticoagulant heparin (36). Furthermore, pretreatment of animals with an antibody to MBP prevents allergen-induced loss of M_2 receptor function and airway hyperresponsiveness (30).

The mechanisms by which eosinophils are selectively recruited to the airway nerves are incompletely understood. The recent demonstration that airway parasympathetic neurons in culture express the selective eosinophil chemoattractant eotaxin (37) may be relevant to this question. Nor is the mechanism of eosinophil activation in the airway nerves understood. However, the finding that NK1 receptor antagonists can prevent allergen-induced M_2 receptor dysfunction without preventing eosinophil recruitment to the nerves may suggest that tachykinins are involved in eosinophil activation (38).

Eosinophils in ozone induced loss of M_2 receptor dysfunction: Likewise, in ozone exposed guinea pigs, there is loss of neuronal M_2 muscarinic receptor function immediately following exposure (19). While exposure to ozone does not increase the number of eosinophils in the bronchiolar lavage, loss of M_2 function also appears to be due to blockade of the receptors by eosinophil major basic protein. Depletion of eosinophils with the antibody to interleukin-5 prior to ozone exposure protects M_2 receptor function and prevents hyperresponsiveness during the 24 hours following

exposure (39). Eosinophil mediated blockade of M_2 receptor function is acutely reversable with heparin. The effects of major basic protein on neuronal M_2 receptor function are short lived, two days after exposure to ozone M_2 receptor function is restored and hyperresponsiveness reversed (figure 1).

Leukocytes in virus-induced M_2 *receptor dysfunction:* Under normal circumstances, most airway viral infections do not cause recruitment of eosinophils. Viral infections generally cause an inflammatory response characterized primarily by neutrophils and mononuclear cells (40). While depletion of inflammatory cells in virus infected guinea pigs protects neuronal M_2 receptor function (27), eosinophils are not the mechanism of dysfunction with viral infection. In contrast to the case with allergen challenge, heparin does not reverse virus-induced M_2 receptor dysfunction. Neither does depletion of eosinophils with the antibody to interleukin-5 protect receptor function (figure 2). Thus, the focus of studies on virus mediated loss of M_2 function has switched to neutrophils and mononuclear cells. While neutrophils and macrophages are known to express neuraminidase (41), which decreases agonist binding to M_2 receptors (see below), whether these cells actually produce any substances, including mammalian neuraminidase, that can act as M_2 receptor antagonists is not yet known.

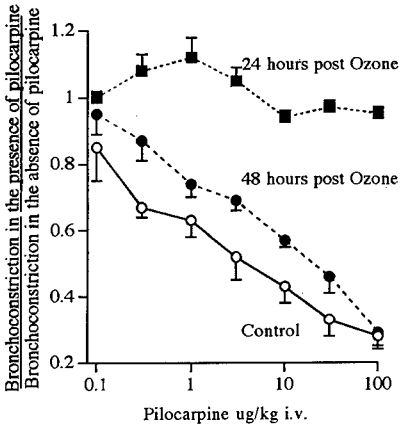

Figure 1

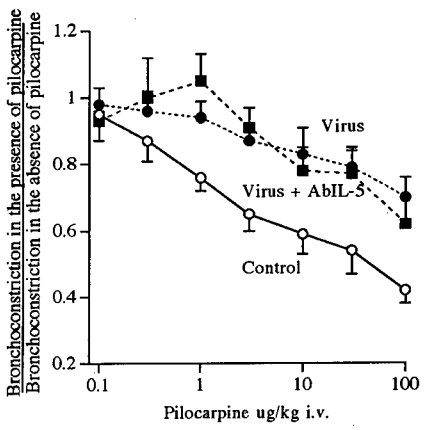

Figure 2

Figure 1: Electrical stimulation of the vagus nerves (2 Hz, 0.2 ms, 5-10V, 22 sec.) causes bronchoconstriction in anesthetized guinea pigs. Pilocarpine inhibits vagally induced bronchoconstriction demonstrating the presence of functional M_2 muscarinic receptors (open circles; n= 6). Immediately after ozone (2 ppm for 4 hours; not shown) and one day (closed squares; n= 5), but not two days (closed circles ; n= 4) post ozone, pilocarpine no longer inhibits vagally induced bronchoconstriction demonstrating loss of neuronal M_2 function only at one day post ozone and recovery at 2 days post ozone.

Figure 2: Pilocarpine inhibits vagally induced bronchoconstriction (see above) in anesthetized guinea pigs demonstrating the presence of functional M_2 muscarinic receptors (open circles; n=5). In virus infected guinea pigs (filled circles; n= 6) pilocarpine no longer inhibits vagally induced bronchoconstriction demonstrating loss of neuronal M_2 function. Depletion of eosinophils with anti-interleukin-5 does not protect M2 receptor function (filled squares; n= 5).

While there are no known M_2 receptor antagonists produced by mononuclear cells, lymphocytes responding to a viral infection produce large amounts of the anti viral cytokine interferon-γ (42). Using cultures of airway parasympathetic neurons, it has been demonstrated that interferon-γ causes both loss of expression of the gene encoding the M_2 receptor and loss of the ability of

muscarinic agonists to suppress release of acetylcholine from the cultured neurons (43). A similar effect can be demonstrated when these cultured neurons are infected with parainfluenza virus directly (43). Thus viral infections may cause loss of M_2 receptor gene expression and function by causing lymphocytes to produce interferon-γ.

It may also be possible for viruses to interact directly with the M_2 receptor on the nerves. The M_2 receptor is heavily glycosylated, and sialic acid residues on the receptor appear to be involved in agonist binding to the M_2, but not the M_3, receptor. Parainfluenza virus contains a neuraminidase that can cleave these residues. When membrane preparations containing M_2 receptors are exposed to parainfluenza virus in vitro, this causes a ten-fold loss of agonist affinity. This effect can be blocked by the neuraminidase inhibitor 2,3-dehydro-2-deoxy-N-acetylneuraminic acid, and can be mimicked by an equivalent concentration of purified *Clostridium perfringens* neuraminidase (44).

Thus, it is possible for viruses to increase vagally-mediated bronchoconstriction by decreasing M_2 receptor gene expression either indirectly, via production of interferon-g, or directly, via effects on the nerves themselves. It is also possible that the virus, by producing neuraminidase, deglycosylates the receptor, decreasing its ability to bind acetylcholine.

Interaction of viral infections with allergen sensitization: While under normal circumstances viral infections do not cause an influx of eosinophils into the airways, the strong association of asthma attacks with both viral infections (45) and airway eosinophilia (46) suggests that this may not be the case in asthmatics. Indeed, viral infections associated with bronchospasm do tend to be associated with airway eosinophilia (47).

Although certain proteins of the respiratory syncytial virus may themselves lead to eosinophilia, the explanation of this association of viral infections with acute airway eosinophilia in asthmatics may also be related to the high incidence of atopy among patients with asthma. It has been demonstrated that while viral infections generally cause both CD4+ and CD8+ lymphocytes to produce interferon-γ, both cell types are also capable of responding to viral infections by producing interleukin-5 (48). This response in CD8+ cells tends to occur in an atopic milieu (i.e., one in which interleukin-4 is present to cause a switch between interferon-g production and interleukin-5 production).

As outlined above, viral infection of guinea pigs normally causes an influx of neutrophils and mononuclear cells, and produces M_2 receptor dysfunction that cannot be reversed by heparin. However, if the animal is first sensitized to a non-viral antigen via intraperitoneal injection, viral infection then causes airway eosinophilia (49). Furthermore, the M_2 receptor dysfunction that occurs in this setting appears to be mediated by eosinophil major basic protein, as it can be acutely reversed by heparin (49).

Conclusion

Ozone exposure, antigen challenge and viral infection are all associated with an influx of inflammatory cells into the lungs. We have demonstrated that at least two inflammatory cells can inhibit neuronal M_2 receptor function. Eosinophilic airway inflammation, as seen in asthmatic airways and after antigen challenge, may directly impair the function of neuronal M_2 receptors in the lungs. Positively charged proteins, especially major basic protein, may act as selective allosteric antagonists at the M_2 receptors. This may account for the ability of the negatively charged molecules heparin and poly-l-glutamate to reverse the effects of antigen challenge. There is at least one other inflammatory cell, as yet unidentified, which also appears to inhibit M2 receptor function. In addition, exposure of the M_2 receptors to inflammatory cytokines, or viral infection of the nerves or can inhibit expression of the neuronal M_2 muscarinic receptors. Greater understanding of these, and other, mechanisms of M_2 receptor dysfunction may lead to the development of new therapeutic strategies in asthma.

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TIOTROPIUM (SPIRIVA™): MECHANISTICAL CONSIDERATIONS AND CLINICAL PROFILE IN OBSTRUCTIVE LUNG DISEASE

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Summary

Inhaled antimuscarinics, often called anticholinergics in clinical medicine, are established as first line bronchodilators in COPD. Tiotropium has been developed as a new generation antimuscarinic following ipratropium. Tiotropium is a specific, highly potent antimuscarinic, demonstrating very slow dissociation from muscarinic receptors. Dissociation from M_2-receptors is faster than from M_3 or M_1, which in functional in vitro studies, appeared as kinetic receptor subtype selectivity of M_3 and M_1 over M_2. The high potency and slow receptor dissociation found its clinical correlate in significant and long lasting bronchodilatation and bronchoprotection in patients with COPD and asthma. In asthma, protection against methacholine challenge exceeded the study period of 48 hours. In COPD, bronchodilatation of about 80% of the plateau was demonstrated after the first dose. Following chronic once daily inhalation for 28 days, the improvement in pulmonary function was sustained and there was a further increase in peak effects, but more importantly a rising baseline, achieving steady state within 2 weeks. Tiotropium achieves very stable long lasting effects with comparatively low variation of bronchodilatation between peak and trough (the level before the next administration). Stable 24 hour effectiveness profiles the compound as the first once daily bronchodilator. Clinical correlates of kinetic receptor subtype selective blockade remain to be shown. Plasma levels of tiotropium at trough are in the low pg/ml range and are unlikely to explain the sustained effectiveness in the airways. Slow dissociation from muscarinic receptors is likely to be responsible for the long duration of action.

Key Words: tiotropium, bronchodilator, COPD, long-acting antimuscarinic, specific muscarinic

Bronchodilators in Chronic Obstructive Airways Disease (COPD)

Considering the physiological importance of muscarinic acetylcholine receptors, the therapeutic use of antagonists is surprisingly limited: pre-medication in anaesthesia and ophthalmology, treatment of gastro-intestinal spasms, urge incontinence, duodenal ulcer, exocrine gland hypersecretion and bronchial smooth muscle relaxation. This constraint is explained by the difficulty to selectively target an organ or tissue. Muscarinic subtype selective agents may not solve this problem, as e.g. smooth muscle and salivary gland are stimulated by the same subtype, the M_3-receptor.

Pharmacotherapy of the lung has the advantage that targeting is possible by inhalational administration. One prerequisite is topical selectivity and quaternary antimuscarinics like ipratropium have low oral systemic absorption, longer pulmonary duration of action and do not penetrate the blood-brain barrier as opposed to the uncharged tertiary analogues e.g. atropine (1). The superior or at least comparable efficacy compared to $beta_2$-agonists (2) combined with fewer side effects (1) has made ipratropium the first line therapy in COPD (3), which is reflected in a prominent place in treatment guidelines of major pulmonary clinical societies (4, 5). On chronic treatment, outlined in a metaanalysis of 1445 patients with COPD by Rennard and colleagues (6), there seems to be an advantage of ipratropium over $beta_2$-agonists in that the former shows an increase in the spirometric baseline. These considerations may explain why an improved anticholinergic/antimuscarinic is very attractive for the treatment of COPD. This report summarizes the preclinical and early clinical observations with the new generation antimuscarinic, tiotropium, and provides some theoretical basis for its observed effects.

Materials and Methods

Human muscarinic receptor studies: Assays were carried out as described (7) using membrane preparations from stably transfected chinese hamster ovary-K1 cells (CHO), expressing the genes for the human muscarinic receptors M_1 to M_5. The apparent dissociation constant K_D was determined from saturation experiments with ^{3}H-tiotropium iodide (1 to 4 h incubation) or ^{3}H-ipratropium iodide (0.5 – 1 h) using equations derived from the law of mass action. The dissociation reaction was studied after receptor–ligand equilibration and addition of 10^{-5} M unlabelled atropine. The dissociation half-life was estimated from the log-linear plot. The dissociation rate constant k_{-1} was calculated by fitting the data to an exponential model. The association reaction was modeled according to Snell (8). Parameter estimation was performed using the NLIN procedure (SAS/STAT). The kinetically determined K_D was calculated as k_{-1}/k_1.

Determination of tiotropium plasma concentrations: The plasma samples were analyzed by HPLC/Ion spray-MS. The limit of quantitation was 4.8 pg/ml of tiotropium bromide. Concentrations below this limit were estimated from the measured values from all dose levels assuming the concentration was proportional to the dose.

Preclinical Results and Discussion

Apparent dissociation constants K_D for tiotropium ranging from 0.1 to 0.3 nM for the subtypes of muscarinic receptors have been reported (7). The affinity for other neurotransmitters is comparatively low (histamine H_1: $K_D = 81$ nM, adrenergic α_1: $K_D = 15$ μM) and unlikely to be of biological relevance. These results demonstrate that tiotropium is a specific and highly potent antagonist at muscarinic acetylcholine receptors M_1 to M_3 (as well as M_4 and M_5 not shown here).

The apparent K_D's calculated from saturation experiments of tiotropium at CHO-membrane preparations expressing the subtypes of human muscarinic receptors seem incubation time dependent: consistent for all 5 subtypes, the values after 1 hour of incubation were 1.5 to 2.8 times higher than K_D's after 4 hours. At M_3-receptors 1 hour incubation resulted 0.45 ± 0.33 and 4 hours 0.25 ± 0.29 (mean ± SD [nM], n = 3). Even after 4 hours, the measured values were not fitted exactly to a one receptor one ligand model curve, indicating a non-equilibrium state. Ipratropium showed no such time dependency. Kinetically determined K_D-values calculated as k_{off}/k_{on} were found to be 24-fold lower for tiotropium, and only 3-fold lower for ipratropium (at M_3, table I).

Tiotropium (0.6 to 6 nM) shifted concentration-effect curves of methacholine in isolated tracheal preparations of guinea pigs dose-dependently to the right, indicating reversible antagonism (7). Comparison of the washout of tiotropium and ipratropium or atropine from guinea pig ciliated cells (7), or guinea pig or human trachea (9) showed a much slower recurrence of induced muscarinic effects following tiotropium. This quality is in agreement with slow dissociation from muscarinic receptors.

TABLE I

Dissociation Constants (k_{-1}/k_1) and Half-Lives of Muscarinic Receptor–Drug Complexes.

	Tiotropium	Ipratropium	Tiotropium	Ipratropium
	K_D [nM]		$t_{1/2}$ [h]	
M_1	0.041 ± 0.038 (4)	0.183 ± 0.042 (3)	14.6 ± 2.2 (5)	0.11 ± 0.005 (3)
M_2	0.021 ± 0.003 (3)	0.195 ± 0.047 (3)	3.6 ± 0.5 (4)	0.035 ± 0.005 (4)
M_3	0.014 ± 0.008 (5)	0.204 ± 0.045 (3)	34.7 ± 2.9 (4)	0.26 ± 0.02 (3)

Means ± SD, ( ) = number of experiments. Membrane preparations from CHO–K1-cells expressing the human muscarinic receptor subtypes. The dissociation constant was kinetically determined at 23°C. The dissociation reaction was initiated by addition of 10^{-5} M atropine.

In guinea pig, as well as human trachea, neuronal acetylcholine (ACh) release is inhibited by prejunctional autoreceptors of the M_2-subtype, whereas tracheal smooth muscle contraction is mediated by M_3-receptors (10). The dissociation half-lives of both tiotropium and ipratropium, showed a slower dissociation from M_3 (8 x) than M_1 (3.5 x) or M_2 (1 x) receptors. The half-lives range from minutes for ipratropium to hours for tiotropium. Therefore, kinetic control of subtype selective blockade, i.e. M_3 and $M_1 > M_2$, is only likely to be shown by tiotropium. In vitro functional evidence of subtype selectivity was provided by Takahashi and colleagues (9). In their experiments tiotropium and atropine equally enhanced electric-field stimulated (EFS) neuronal ACh-release from guinea pig trachea, but also blocked the neuronal cholinergic smooth muscle contraction. This enhancement of ACh-release was lost two hours after start of washout of both test drugs, at a time when airway smooth muscle neuronal cholinergic contraction was still blocked by tiotropium, but no longer by atropine. Whether subtype selectivity will be translated into higher efficacy or improved safety remains to be demonstrated in clinical trials.

Tiotropium provided long lasting protection against methacholine induced bronchospasm in dogs and guinea pigs (7). The preclinical safety of this new antimuscarinic was demonstrated in toxicological studies with inhalation, oral, or intravenous administration of up to 2 years duration.

Human Pharmacology

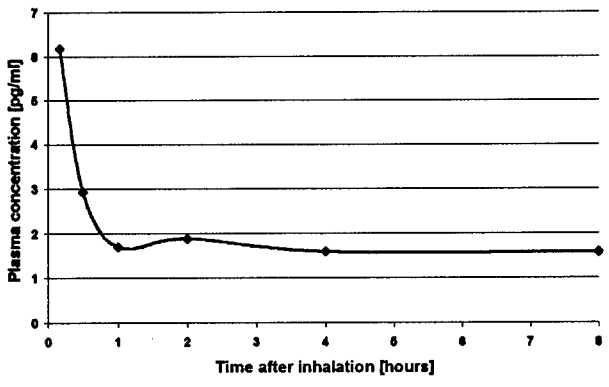

Fig. 1.
Calculated geometric mean plasma concentrations standardized to a target dose of 10 µg tiotropium bromide.

Several single and multiple dose escalating trials with tiotropium were conducted in normal volunteers. There were no relevant drug-related effects on vital signs (systolic and diastolic blood pressure, pulse and respiratory rate), electrocardiography, pupillometry, or routine laboratory parameters. Dry mouth, as a typical systemic antimuscarinic effect, was reported after higher doses. After inhalation of tiotropium, peak plasma levels attained a maxi-

mum five minutes post dose with subsequent rapid decline in less than 1 hour to very low levels (in the 2 pg/ml range, fig. 1). At this low level, plasma tiotropium was eliminated with a terminal phase half-life of 5-6 days, independent of the dose.

Tiotropium in Asthmatic Patients

The clinical duration of action of tiotropium was studied in a randomized double-blind 5-period cross-over study including 12 mild asthmatic patients with documented hyperresponsiveness to inhaled methacholine by O'Connor and colleagues (11). Inclusion criteria were a provoking concentration causing a 20% fall in FEV_1 (PC_{20}) of less than 8 mg/ml, but a baseline FEV_1 > 80% of the predicted normal value. Inhaled sympathomimetics and caffeinated beverages were withheld for at least 8 hours before each study period. The study periods were: placebo and tiotropium bromide (10, 40 and 80 µg single dose) administered as a powder in a lactose carrier via the breath-activated Inhaler Ingelheim™. The washout period was 8 to 25 days. Pulmonary function and PC_{20} was assessed for 48 h (72 h in 4 patients).

TABLE II

% Increase of FEV_1 (Mean ± SEM) Compared to Placebo

Time\Tiotropium:	10 µg	40 µg	80 µg	Active vs. placebo $p < 0.05$. At 36 and 48 h after drug administration FEV_1 did not differ significantly from placebo.
2 h	6.7 ± 3.4	8.2 ± 3.9	10.0 ± 2.7	
12 h	10.4 ± 4.1	5.5 ± 4.8	11.1 ± 3.9	
24 h	7.3 ± 3.5	7.1 ± 3.8	9.4 ± 3.7	

Tiotropium caused a mild but statistically significant bronchodilation in a time-dependent but not dose-dependent manner (table II). This patient group of mild asthmatics, on average, reacted to less than 1 mg/ml methacholine. Tiotropium provided clinically significant protection (increase of at least one doubling concentration) against the methacholine challenge in 11 of 12 patients at 10 µg and in all patients at 40 or 80 µg (table III). This protection was sustained for up to 48 hours in a dose and time-dependent manner and was still present in 9 of 12 patients, even at the 10 µg dose level. Complete protection against the highest concentration of methacholine (128 mg/ml) was achieved in most patients with the two higher doses of tiotropium.

TABLE III

Clinically Significant or Complete Protection Against Methacholine Challenge in Patients with Mild Asthma

	Clinically significant */ complete protection**:				
	2 h	12 h	24 h	36 h	48 h
Placebo	/0	/0	/0	/0	/0
Tiotropium bromide, 10 µg:	11 / 2	11 / 1	10 / 0	10 / 0	9 / 0
Tiotropium bromide, 40 µg:	12 / 8	12 / 4	12 / 4	12 / 1	10 / 0
Tiotropium bromide, 80 µg:	12 / 11	12 / 7	12 / 5	12 / 2	11 / 2

Number of patients out of 12 who showed clinically significant protection*, i.e. 20% fall in FEV_1 at one doubling concentration higher than placebo, or complete protection**, i.e. less than 20% fall in FEV_1 after challenge with the highest dose of methacholine, 128 mg/ml.

Tiotropium in Patients with COPD

Single dosing studies: The bronchodilator response and the safety of tiotropium in patients with COPD was studied in a pilot dose–response study (12). The results were similar to a subsequent larger double-blind placebo-controlled crossover study by Maesen and colleagues (13). All patients conformed to the American Thoracic Society definition of COPD (14). Patients (n=35,

mean baseline FEV_1 = 1.34 l, 44% of predicted value) inhaled single doses of 10, 20, 40 and 80 µg tiotropium bromide or placebo from the powder inhaler. The washout period was 72 hours.

Significant bronchodilation, as determined by FEV_1 improvement, was confirmed at all doses. The effect was dose-dependent, the 10 µg producing about half the improvement in FEV_1 of the 80 µg dose. The effect was rapid, observed already at the first assessment point of 15 minutes. The duration of effect extended to the 32 hour observation period, especially with the doses of 20 µg and higher. A carry-over effect was observed, particularly at the 80 µg dose. This was evident by a relatively large placebo response since the washout time was only 72 hours. In a second series of analysis, the patients having received more than 10 µg in the previous period were excluded, which led to greater separation between tiotropium and placebo (fig. 2). The peak FEV_1 ranged 20 – 25 % above test-day baseline for active treatments compared to 11 % for placebo. There was considerable circadian variation with a night/early morning dip of spirometric performance. During this period, 16 to 24 hours after administration, the difference in FEV1 between active treatment and placebo became smaller. These dips spontaneously resolved and interestingly, dose dependent differences between active and placebo recurred. This indicates that the morning dip has contributing factors beyond the cholinergic tone.

The drug was well tolerated at all doses and no abnormalities were observed in routine laboratory assessments or clinical evaluation. Reported dyspnoea was reduced in the active treatment groups (table IV).

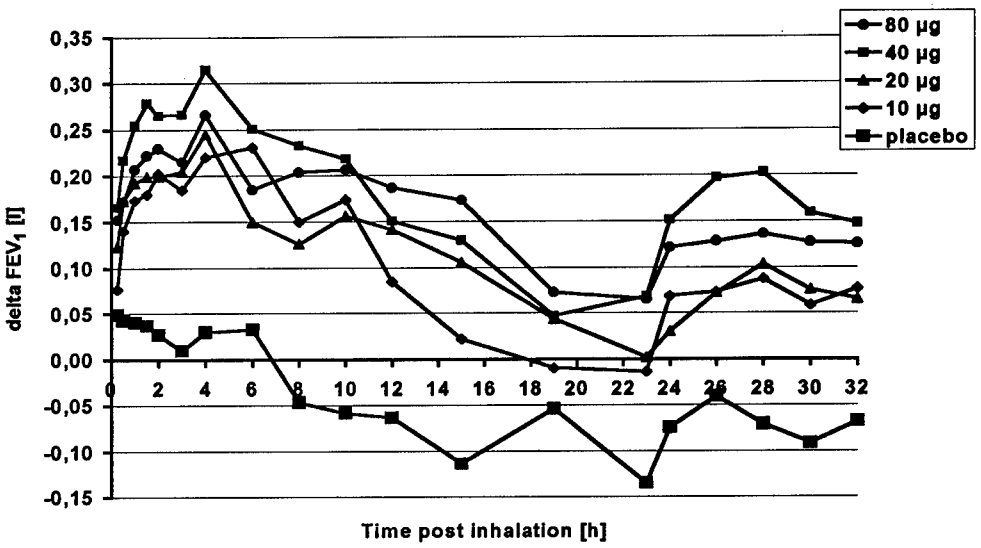

Fig. 2.
Increase in adjusted mean FEV_1 from test day baseline, excluding test days which followed tiotropium 20, 40 or 80 µg. Adapted from (13).

TABLE IV

Patients Reporting the Most Frequent Adverse Event, Dyspnoea, Mainly Reflecting the Patient's COPD Condition.

Placebo	Tiotropium 10 µg	Tiotropium 20 µg	Tiotropium 40 µg	Tiotropium 80 µg
15 of 35 (43%)	8 of 34 (24%)	9 of 34 (27%)	6 of 34 (18%)	5 of 34 (15%)

Repeated dosing studies: An appropriate dose for such a long acting drug can only be defined by steady state measurements. Patients with COPD (n=169, mean baseline FEV_1 = 1.08 l,

42% of predicted value) were randomized to receive placebo, 4.5, 9, 18 or 36 μg tiotropium by dry powder inhaler once daily for 28 days in a double blind, parallel group comparison (tiotropium doses from now on refer to the base, not the salt form). Spirometry was assessed at 8.00, 10.00 and 12.00 at weekly visits during baseline (2 weeks), treatment (4 weeks) and post-treatment (3 weeks) periods. Drug was inhaled immediately after the 12.00 noon lung function tests and then measured for 6 hours.

Preliminary results (15) showed that all doses of tiotropium were significantly more effective than placebo (FEV_1 and PEFR) showing increased effects with increasing dose, but no statistical difference among the doses. These improvements in lung function, clinically and statistically significant, were seen within 1 hour following the first dose and were maintained over 24 hours. All doses produced increases in FEV_1 that were significantly greater than placebo ($p<0.05$) at both the start and end of the 24 hour dosing interval. The 24 hour bronchodilatory effectiveness was maintained throughout the 28 days. After termination of drug administration the decline of improved pulmonary function towards baseline lasted more than one week in the higher dose groups. Only two serious adverse events were reported, neither considered by the investigator as drug-related. No significant changes were observed in routine laboratory parameters and ECGs. The most common antimuscarinic side effect, patients reporting dry mouth, was slightly increased in the highest dose group (placebo: 0 of 35; tiotropium 4.5 μg: 2 of 34; 9 μg: 0 of 33; 18 μg: 2 of 33; 36 μg: 3 of 34, always mild, except one patient in 36 μg group with moderate symptoms). Plasma levels in steady state could mainly be assessed at peak (5 minute values) and at the trough only at the highest dose (table V).

TABLE V

Plasma Levels after 28 Days of Once Daily Inhalation of Tiotropium.

Tiotropium dose:	4.5 μg	9 μg	18 μg	36 μg	
Plasma level at peak (trough), [pg/ml]:	b.l.q.	9.4	14.0	45.3	(4.0)

b.l.q. = below level of quantitation.

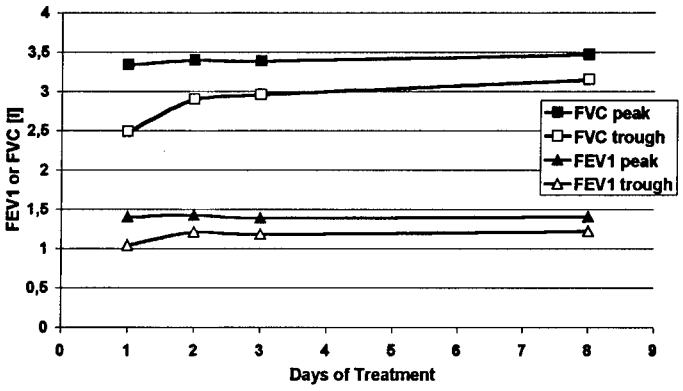

Fig. 3.

Onset of pharmacodynamic steady state following once daily early morning inhalation of tiotropium in 17 patients with COPD (16).

In a subgroup of a long-term (1 year) trial, 17 patients with COPD underwent frequent pulmonary function testing to characterize the onset of the pharmacodynamic steady state (16). The major proportion of spirometric improvement at peak was achieved within 24 hours (fig. 3). Increases in trough FEV_1 (before next dose) and, more so, trough FVC were pronounced on the second day and further improvement still evident after one week into therapy. The difference between trough and peak became smaller as the study progressed.

Mechanistic Considerations of the Duration of Action

Tiotropium has a duration of action by far exceeding 24 hours and at a low approximately 2 pg/ml plasma level, a terminal phase half-life of 5 – 6 days. Does the plasma level control the pharmacodynamic activity? Following inhalation of 20 µg tiotropium bromide an estimated 4 µg may reach the lungs given the fine particle fraction (< 5.8 µm) being 20 %. Deposition in the lungs will be followed by dissolution in the epithelial lining fluid (ELF). Using an estimate of 20 ml for ELF (17, 18) gives a concentration of 2 µM, which is 5 orders of magnitude higher than the kinetically determined K_D of 14 pM. From the lungs, there is rapid absorption into the circulation with rapid decline to very low levels (fig. 1). In steady state, the 18 µg dose resulted a 5 min plasma level of 14 pg/ml (= 30 pM). The trough steady state plasma level of the 18 µg dose can only be estimated from the 36 µg dose, assuming dose proportionality, as 2 pg/ml (= 4.2 pM).

Tiotropium should be freely diffusible and the plasma concentration is likely to be accessible to receptors on the cell membrane. Using the following equation, derived from the law of mass action, the receptor occupancy can be estimated as a function of the drug concentration:

$$[RD] / [R_t] = [D] / (K_D + [D])$$

In this equation [RD] is the concentration of receptor-drug complex, $[R_t]$ of the total number of receptors and [D] of the concentration of free drug. $[RD]/[R_t]$ reflects the proportion of occupied receptors. Two curves were constructed, one using the kinetically determined K_D of tiotropium at M_3-receptors of 0.014 nM, and the second using the apparent K_D of 0.45 nM determined after 1 hour incubation (fig. 4).

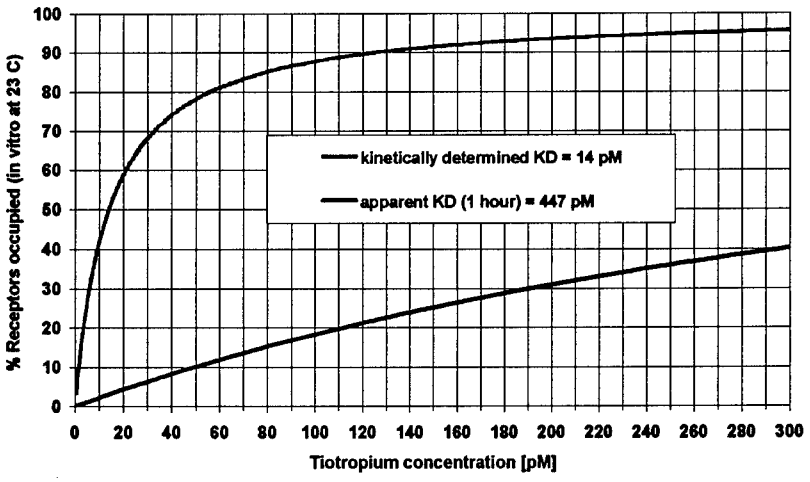

Fig. 4.
Calculated receptor occupancy dependent from drug concentration using the kinetically determined K_D or the apparent K_D. Formula used see text.

Assuming an equilibrium state, then the curve constructed with K_D = 14 pM may apply: the trough concentration of 4.2 pM can maximally correspond to 23% occupancy of muscarinic receptors, probably less, because binding was measured at 23°C, not 37°C (higher K_D at 37°C). The curve constructed with the apparent K_D = 447 pM is adequate to investigate receptor occupancy at non-equilibrium (which is the case when concentrations rapidly declinine from peak to trough): in this case, the trough concentration of 4.2 pM tiotropium corresponds to 1 % receptor occupancy and the systemic peak levels of 30 pM to only 6 %. Peak concentrations of 9 nM (4.2 ng/ml) would be needed to achieve 95 % receptor occupancy. These high concentrations are likely be reached only topically in the lungs, as estimated above.

The link between free receptors, receptor occupancy with agonist and effects can presently only be derived from in vitro experiments: Ringdahl (19, 20) described 0.36 to 7 % receptor occupancy by the agonist as necessary for a 50 % response (value compound specific, i.e. contraction of guinea pig ileum by oxotremorine analogues). Given the high postjunctional neurotransmitter tonus the agonist ACh is likely to still elicit a nearly full cholinergic response if less than 50% of the receptors are occupied by the antagonist.

In conclusion, the trough plasma levels of tiotropium are unlikely to provide functionally relevant systemic occupancy of muscarinic receptors in agreement with a relatively low incidence of systemic antimuscarinic side effects in the clinical trials. The systemic peak levels with the short exposure time of minutes, are also unlikely to induce relevant occupancy, but the much higher topical lung levels achieve a high receptor occupancy. Plasma levels do not explain the long duration of action. The very slow dissociation from muscarinic receptors is likely to control the bronchodilating duration of action of tiotropium.

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AUTOANTIBODIES AGAINST M_2 MUSCARINIC RECEPTORS IN PATIENTS WITH CARDIOMYOPATHY DISPLAY NON-DESENSITIZED AGONIST-LIKE EFFECTS

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Summary

Circulating autoantibodies against the human M_2 muscarinic receptors have been previously shown in 38% of patients with idiopathic dilated cardiomyopathy. The functional properties of these autoantibodies are reported herein. They were able to decrease the cell beating frequency of myocytes in cultured neonatal rat heart cells in a dose-dependent manner without desensitization over a period of more than 5 hours whereas the non-specific muscarinic receptor agonist carbachol also inhibited the heart cell beating frequency but was desensitized within 1 hour. In the same cell culture, anti-M_2 muscarinic receptor autoantibodies were not able to induce internalization of muscarinic receptor whereas carbachol did. These results demonstrate for the first time that anti-M_2 muscarinic receptor autoantibodies from patients with idiopathic dilated cardiomyopathy have stimulatory muscarinic activity *in vitro,* which differ from normal muscarinic agonists by non-desensitization.

Key Words: muscarinic receptor, autoantibody, cardiomyopathy

Idiopathic dilated cardiomyopathy (DCM) is defined as heart muscle disease of unknown aetiology and is one of the main causes for severe heart failure and the most common cause for heart transplantation. An autoimmune mechanism is very likely to play an important role in the pathogenesis of DCM. We have previously demonstrated the presence of circulating autoantibodies against the human M_2 muscarinic receptors in 38% of patients with DCM (1, 2). It was found that the autoantibodies were able to interfere with the muscarinic receptor ligand binding on rat heart membrane (1, 2). However, it is still not known whether these autoantibodies have any functional relevance and could thus contribute to the pathogenesis or symptomatology of DCM. Experimental approach was made to raise rabbit anti-peptide antibodies against the same target peptide as recognized by DCM anti-M_2 muscarinic receptor autoantibodies. Results revealed that these antibodies displayed agonist-like muscarinic receptor activity as shown by decreases in heart rate, cardiac output, left ventricular systolic pressure and maximal rate of rise of ventricular pressure in a dose-dependent manner *in-vivo* (3) and a decrease in isoproterenol-stimulated adenosine 3′, 5′-cyclic monophosphate (cAMP) production *in-vitro* (4). The functional properties of these rabbit antibodies may however differ from human DCM autoantibodies. The main purpose of this study was therefore to examine the functional activity of anti-M_2 muscarinic receptor autoantibodies from patients with DCM.

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Methods

Patient serum samples

Sera were obtained from the same population of patients with DCM admitted to the Department of Cardiology, Kanazawa Medical University, Kanazawa, Japan, as used in the previous work (2).

Peptide

A peptide corresponding to the sequence of the second extracellular loop of human M2 muscarinic receptors, VRTVEDGECYIQFFSNAAVTFGTAI, was synthesized by Vetrogen Inc. (London, Canada) and used for determination of positivity of patient sera by an enzyme-linked immunoabsorbent assay as previously described (2).

Antibody purification

The anti-M2 muscarinic receptor autoantibodies were further purified from sera of patients with positive autoimmune reactivity using affinity-chromatography on the M2 receptor peptide.

Chronotropic assay and binding experiments
Myocytes were cultured using 1- to 2-day-old Wistar rats according to the previously described method (5). Chronotropic assays were performed on monolayers of cultured neonatal cardiomyocytes as previously described (5-6). Binding experiments were done with [^{3}H]-NMS, (23pM - 2.7nM), on cultured neonatal cardiomyocytes for 1 h. The specific binding of [^{3}H-NMS] was determined as the amount of binding inhibited in the presence of 1 μM atropine. The effects of antibodies and muscarinic ligands were observed when these reagents were preincubated with cultured cardiomyocytes for 3 hours before binding studies were performed.

Results

The chronotropic studies demonstrated that the autoantibodies purified from DCM patients (n=6) were able to decrease the heart cell beating frequency in both a dose-dependent and time-dependent manner (Fig. 1-2). The negative chronotropic effect of the autoantibodies was sustained for more than 5 hours and was persistent even after washing and subsequent incubation for a few hours in fresh culture medium without the autoantibodies. This negative chronotropic effect was, however, completely and rapidly reversed by addition of the muscarinic receptor antagonist atropine (1 μM) (Fig. 2).

The hydrophilic muscarinic ligand NMS was used to study the internalization of the muscarinic receptors. The binding results showed that after preincubating cultured cardiomyocytes for 3 hours with carbachol (100 μM), the number of NMS binding sites (Bmax) ($4.2 \pm 0.7 \times 10^{-12}$ M) decreased by 57% as compared with the control (in the absence of carbachol, $9.9 \pm 1.7 \times 10^{-12}$M). However, no such decrease in NMS binding sites was found in autoantibody-treated cardiomyocytes ($11.9 \pm 2.2 \times 10^{-12}$M). The affinity constant of NMS in both groups remained unchanged.

Discussion

Circulating autoantibodies against human M2 muscarinic receptors have been repeatedly demonstrated in a subgroup of DCM patients (1, 2). It appears that these anti-muscarinic receptor autoantibodies are present mainly in patients with DCM but not in hypertrophic cardiomyopathy and hypertension (2), suggesting that these anti-muscarinic receptor autoantibodies may play a role, at least in part, in the pathogenesis of DCM. However, a detailed characterization of the functional properties of these human autoantibodies is still lacking. Therefore, it is the main

purpose of this study to characterize the functional properties of anti-human M2 muscarinic receptor autoantibodies from patients with DCM.

The chronotropic assay in presence of a muscarinic receptor ligand or agent is generally accepted as one of the most important functional parameters for muscarinic activity. Chronotropic studies of these human anti-muscarinic receptor autoantibodies demonstrated that they were able to

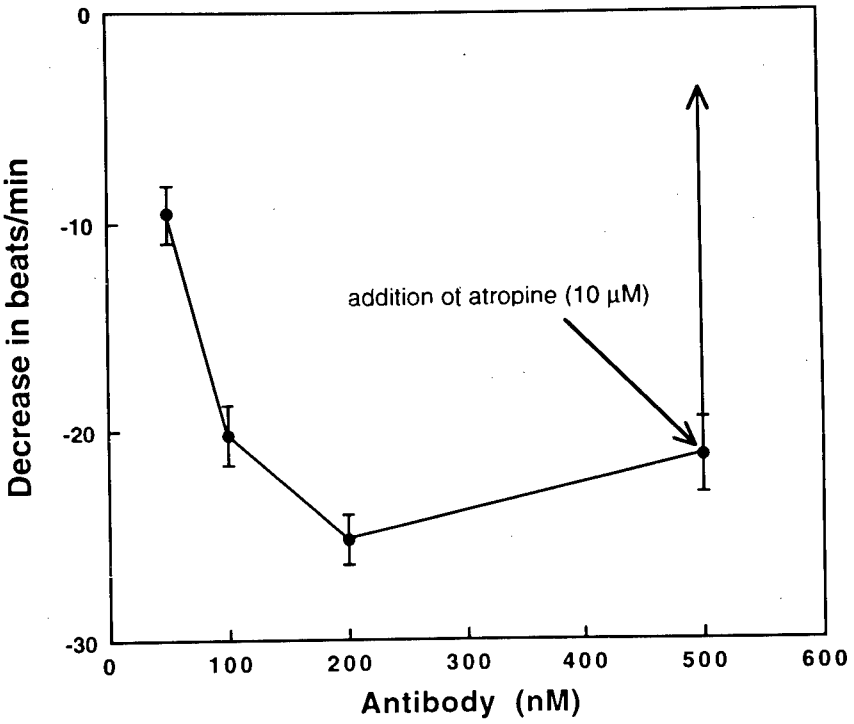

Fig. 1.
Dose-dependent negative chronotropic effect of autoantibodies on cultured neonatal rat cardiomyocytes. atropine: 1 μM. Data are shown as mean ± sem of 25 experiments.

decrease the heart cell beating frequency in a specific manner. This negative chronotropic effect was rapidly reversed by atropine, suggesting that the chronotropic effect induced by the autoantibodies was specific to the muscarinic receptor. This is in contrast with observation in which the negative chronotropic action by muscarinic receptor agonist carbachol (100 μM) was subject to relatively rapid desensitization, as shown by the fact that the negative chronotropic effect of carbachol waned after 1 hour in its continued presence. These results indicate that anti-heart muscarinic receptor autoantibodies have stimulatory muscarinic receptor activity *in-vitro,* which differs from that of normal muscarinic agonists by its non-desensitization.

The hydrophilic muscarinic ligand NMS was usually used to study the internalization of the muscarinic receptors in cultured neonatal cardiomyocytes. The ligand binding studies showed that after preincubating cultured cardiomyocytes for 3 hours with carbachol, the number of NMS binding sites decreased by 57%. However, no such decrease in NMS binding sites was found in autoantibody-treated cardiomyocytes, suggesting that carbachol can induce a decrease in plasma-

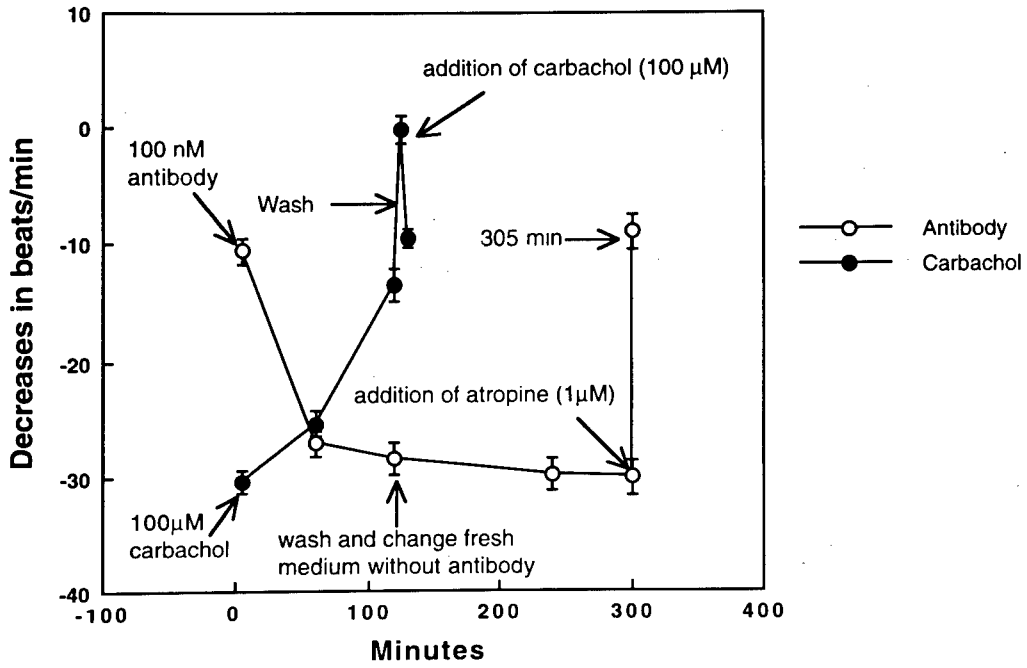

Fig. 2.
Time-dependent negative chronotropic effect of autoantibodies (100 nM) or carbachol (100 µM) on cultured neonatal rat cardiomyocytes. atropine: 1 µM. Data are shown as mean ± sem of 40 experiments.

membrane-localized muscarinic receptors whereas the autoantibody had no such effect. It could be that cross-linking of receptors by the bivalent autoantibodies inhibit the internalization of the receptors in the same manner as has been reported for bivalent lectins.

In summary, we report here for the first time to our knowledge that autoantibodies purified from patients with DCM are able to exhibit a stimulatory muscarinic activity which differs from that of normal muscarinic agonists by its resistance to desensitization. This might be explained, at least in part, by the fact that autoantibodies cannot induce internalization of the receptors as receptor internalization has been generally accepted as one of the main mechanisms for desensitization in heart failure. These results add weight to our hypothesis that anti-cardiac receptor autoantibodies in patients with DCM are of pathogenic importance. This has been strengthened further by our parallel study which showed that active immunization of rabbits with M2 muscarinic receptor peptide as used in the present study, once a month during one year, was able to induce cardiomyopathy-like pathological changes (7).

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MUSCARINIC CHOLINERGIC SIGNALING IN CARDIAC MYOCYTES: DYNAMIC TARGETING OF M2AChR TO SARCOLEMMAL CAVEOLAE AND eNOS ACTIVATION

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Summary

The isoform of nitric oxide synthase (eNOS or NOS3) originally described in endothelial cells is also expressed in a number of other cell types, including cardiac myocytes. eNOS is activated in both atrial and ventricular myocytes, including specialized pacemaker cells, by M2AChR agonists, among other stimuli. In cardiac myocytes, as in endothelial cells, eNOS is targeted to sarcolemmal caveolae, due to both co-translational myristoylation and later palmitoylation, and by the presence of a caveolin binding domain in eNOS which interacts with the caveolin scaffolding domain. In the absence of ligand, the M2AChR is not associated with caveolar microdomains, but translates into caveolae upon agonist (but not antagonist) binding. Finally, the role of M2AChR-induced eNOS activation in regulating $I_{Ca\text{-}L}$ via activation of guanylyl cyclase has been confirmed in ventricular myocytes of mice that lack functional eNOS (i.e., $eNOS^{null}$).

Key Words: eNOS, M2AChR, cardiac myocytes, muscarinic cholinergic signaling

Nitric oxide (NO) plays a number of important roles within the cardiovascular system since its original identification as endothelium-dependent relaxing factor (EDRF). Within the heart, NO acts to mediate muscarinic and purinergic receptor signaling in cardiac myocytes and, after generation of the inducible isoform of nitric oxide synthase (iNOS), as an effector of innate immunity.

eNOS in the Heart

The principal source of NO within the normal myocardium is the endothelium of the coronary vasculature and endocardium. Endothelial cells constitutively express the NOS isoform termed eNOS, which, as in other organs and tissues, generates NO in response to specific extracellular signals to regulate vascular smooth muscle tone, vascular permeability, and

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platelet adhesion, among other actions. In addition to coronary vascular and endocardial endothelium, both atrial and ventricular myocytes — including specialized pacemaker tissue such as sinoatrial and atrioventricular nodal cells — express eNOS as well, the activation of which is also dependent on specific intracellular and extracellular signals (see 1,2 and references therein). As in endothelial cells, eNOS is targeted in cardiac myocytes to specialized signal transduction domains termed caveolae in plasma membranes and, in the case of cardiac myocytes, possibly T-tubular membranes as well (discussed in greater detail below) (3,4).

Also, as in endothelial cells, eNOS targeting to caveolae in cardiac myocytes requires co-translational myristoylation and subsequent palmitoylation for efficient targeting of the enzyme to the specialized lipid microdomains characteristic of caveolae (reviewed in 4). Although eNOS also contains a caveolin binding motif, this appears to be insufficient for correct targeting of eNOS to caveolae in the absence of acylation. The scaffolding domain on caveolin-3, the myocyte-specific caveolin, binds to a ϕxxxxϕxxϕ motif (ϕ are the amino acids Trp, Phe or Tyr) in the catalytic domain of eNOS (FSAAPFSGW) which locks the receptor in an inactive conformation (5). Indeed, Sessa and his colleagues (6) have demonstrated that site-directed mutagenesis of the aromatic amino acid moieties in eNOS caveolin binding motif results in the inability of caveolin to inhibit eNOS activity. As detailed above, eNOS targeting to caveolae, however, appears to be independent of its association with caveolins (4), with correct targeting to caveolar microdomains requiring acylation.

Although not yet formally demonstrated in cardiac myocytes, it is presumed that eNOS in myocytes participates in a Ca^{2+}-calmodulin-caveolin regulatory cycle similar to that described in endothelial cells. When the Ca^{2+} concentration increases in the vicinity of caveolae containing eNOS, Ca^{2+}-activated calmodulin removes eNOS from its inactive heterodimer conformation with caveolin (caveolin-3 in cardiac myocytes), with subsequent activation of eNOS (4). With the decline of Ca^{2+} towards basal levels, calmodulin dissociates from eNOS, and eNOS reassociates with caveolin (a process believed to be facilitated by palmitoylation).

Although it appears clear that myocyte eNOS is targeted to sarcolemmal caveolae, the possibility that eNOS might also be localized within the T-tubular system may have important consequences for the regulation of excitation-contraction coupling. Over 30 years ago, Ishikawa (7) hypothesized that the development of the T-tubular system in fetal cardiac muscle occurred by successive fission and coalescence events among caveolae, that began with a single caveola or clusters of caveolae at the sarcolemmal membrane. It had been known that the lipid composition of the T-tubular system — enriched in glycosphingolipids and cholesterol with a low phospholipid content — was similar to that of caveolae. Recently, using antibodies to caveolin-3 — the muscle-specific caveolin — Parton and his colleagues (8) confirmed that the T-tubular system in both skeletal and cardiac muscle contains caveolin-3. Interestingly, while caveolin-3 disappears from T-tubular membranes in skeletal muscle after development, caveolin-3 expression in T-tubules is sustained in cardiac muscle.

The potential localization of eNOS to T-tubular membranes, in addition to its targeting to sarcolemmal caveolae, is of interest in the context of recent work by Meszaros and colleagues (9,10), and by Stamler and co-workers (11) on the effects of NO on the cardiac ryanodine receptor Ca^{2+} release channel (CRC), which is localized to T-tubular membranes. Building on their original observations with the skeletal muscle CRC, in which Meszaros et al. (9) demonstrated that NO decreased CRC activity by a mechanism that appeared to be independent of cGMP generation, this laboratory went on to document a similar response in

CRC in canine cardiac muscle-derived sarcoplasmic reticulum microsomes (10). Importantly, they demonstrated regulation of channel activity both with NO donors and by activation of NOS activity within the microsomal preparations (presumably eNOS, since eNOS was detectable by western blot in these microsomal fractions). These authors concluded that eNOS in cardiac muscle, which, like the CRC, is a Ca^{2+} activated protein, may act to downregulate CRC activation during muscle depolarization. This interpretation is supported by preliminary data from this group that activation of eNOS in intact cells suppresses the frequency and duration of Ca^{2+} "sparks" generated in intact cardiac myocytes (12). The underlying biochemistry is complex, however. This is highlighted in the work of Xu et al. (11) who, also working in CRC purified from canine cardiac muscle, found that S-nitrosothiol NO donors result in reversible activation of CRC activity when measured after their incorporation into plasma lipid bilayers.

Caveolar Targeting of M2AChR and eNOS in Cardiac Muscle

eNOS, in cardiac myocytes, is activated by muscarinic cholinergic and purinergic agonists (13-15). An increasing number of reports support the concept that M2AChR and A_1 purinergic receptor control of $I_{Ca\text{-}L}$ in some cardiac myocyte phenotypes is dependent on activation of myocyte eNOS (reviewed in 2). Indeed, the original report by Balligand et al. (16), that indicated that cardiac myocytes might express a "constitutive" isoform of nitric oxide synthase, was based on their observations that suppression of the spontaneous beating rate of neonatal ventricular myocytes by carbachol could be blocked by a NOS antagonist. The principal subcellular target of NO generated by eNOS in cardiac myocytes is presumed to be the heme moiety in soluble guanylyl cyclase, although, in addition to the calcium release channel noted above, several non-cGMP-mediated effects of NO on $I_{Ca\text{-}L}$ have also been demonstrated (17). However, the importance of these "direct" effects of NO on the physiological regulation of this and other ion channels remains to be determined.

The net effect of an increase in myocyte intracellular cGMP will depend on the extent of activation of adenylyl cyclase and the activities of potential downstream target proteins (e.g., protein kinase G, cGMP-regulated PDEs [i.e., types 2 and 3]) and the activity of cGMP-hydrolyzing PDEs (i.e., PDEs 3 and 5). A small increase in myocyte cGMP — whether the result of increased NO delivered by a nitrovasodilator or eNOS activation in myocytes or adjacent endothelium — could enhance cAMP levels by inhibiting PDE 3 activity. Larger amounts of NO would lead to larger increases in cGMP levels, thereby decreasing cAMP content (by activating a type 2 PDE) and inhibiting downstream cAMP signaling (by activating a protein kinase G). Recent work from our laboratory, using single cell reverse transcription polymerase chain reaction, has identified both type 2 and type 3 PDEs in adult rat ventricular myocytes (X. Han, S.D. Wiviott, Y. Zhao, D.J. Opel, R.A. Kelly, manuscript under review).

While many of the details of the Ca^{2+}/calmodulin-eNOS regulatory cycle within caveolae are now understood, the sequence of events leading to Ca^{2+} influx into caveolar microdomains remains unclear. Some G protein-coupled receptors, such as endothelin receptors, appear to be constitutively present within caveolae, but this is clearly not the case for a number of other G protein-coupled receptors, including the M2AChR (5). Using two complementary approaches, density gradient (isopycnic) centrifugation following detergent-free preparation of adult rat ventricular myocyte lysates, and co-immunoprecipitation protocols using antibodies to caveolin-3, eNOS and the M2AChR, we demonstrated that eNOS and caveolin-3 were present in caveolar fractions in the absence of muscarinic cholinergic agonists, but the M2AChR was not (18). Following exposure to the muscarinic agonist carbachol — but not

the antagonist, atropine — the M2AChR (tracked by [^{3}H]QNB binding) moved into myocyte caveolar microdomains. Moreover, in the presence of agonist (but not antagonist), the M2AChR could be co-immunoprecipitated by anti-caveolin-3 antibodies (18). The relevance of this intracellular trafficking of ligand-M2AChR complexes into caveolae to receptor desensitization remains controversial. Nevertheless, it appears that translocation into caveolae is essential for signaling.

Recent evidence obtained from ventricular myocytes of mice with targeted disruption of the eNOS gene (i.e., eNOS-/- mice) indicates that the lack of functional eNOS interrupts muscarinic cholinergic control of $I_{Ca\text{-}L}$ in these cells (19). eNOS-/- mice are hypertensive and develop cardiac hypertrophy as they age, and these animals have also been shown to exhibit an accelerated degree of vascular remodeling in response to injury (20-22). We isolated atrial and ventricular myocytes from neonatal and adult eNOS-/- animals and their eNOS+/+ littermates. Compared to wild-type (WT) ventricular myocytes, eNOS-/- myocytes exhibited little or no suppression of isoproterenol-induced increases in $I_{Ca\text{-}L}$ contractile amplitude in response to muscarinic cholinergic agonists (e.g., carbachol; Figure 1) (19). The absence of an effect on M2AChR-control of $I_{Ca\text{-}L}$ in ventricular myocytes from eNOS-/- animals was reflected in the absence of any increase in cGMP levels in response to carbachol in eNOS-/- cells. However, cGMP levels did increase in eNOS-/- cells as in WT myocytes in response to the NO donor SIN-1, indicating that a guanylyl cyclase was present in the eNOS-/- cells, if functionally uncoupled from M2AChR signaling. Importantly, M2AChR signaling pathways that are *not* mediated by cGMP remained intact in eNOS-/- cells, such as the activation of the acetylcholine-sensitive K^+ current $I_{K(Ach)}$.

We have recently confirmed and extended these observations in neonatal murine ventricular myocytes, which beat spontaneously in primary culture. Neonatal ventricular myocytes from eNOS+/+ animals respond to isoproterenol with an increase in beating rate, which can be suppressed with muscarinic cholinergic agonists, while myocytes from eNOS-/- fail to respond to cholinergic agonists. However, in preliminary data from our laboratory, the responsiveness of eNOS-/- neonatal myocytes to carbachol could be restored by transfection with wild-type eNOS, but not with a myristoylation-deficient eNOS. We also tested whether increased expression in neonatal rat ventricular myocytes (which have functional eNOS) of a peptide containing the caveolin-3 scaffolding domain would interrupt normal muscarinic signaling via eNOS in these cells. As expected, transfection with this peptide (but not a control peptide with a similar amino acid composition) abrogated M2AChR control of beating rate in the presence of a sympathetic agonist. These reconstitution experiments confirm both the essential role of eNOS in coupling M2AChR signaling to the control of $I_{Ca\text{-}L}$ and myocyte automaticity and the importance of eNOS subcellular localization within caveolae in mediating this signal transduction pathway (O. Feron, C. Dessy, D.J. Opel, M.A. Arstall, R.A. Kelly and T. Michel, manuscript under review).

Finally, diminished eNOS expression and activity in myocytes, due to inflammatory cytokines or to agents that cause sustained elevations in intracellular cAMP (such as catecholamines or phosphodiesterase inhibitors such as milrinone), may have important consequences for parasympathetic nervous system regulation of myocardial electrophysiology and contractile function. Sustained increases in cAMP directly diminish eNOS transcription as well as interrupt post-translational processing and targeting of eNOS to sarcolemmal caveolae (23,24). Indeed, this decreased expression of myocyte eNOS by drugs that elevate cAMP would be predicted to diminish parasympathetic nervous system control of $I_{Ca\text{-}L}$, and perhaps contribute to the generation of cardiac arrhythmias. With reference to human disease, heart failure is characterized by high levels of sympathetic nervous system tone and is often treated,

during short-term decompensation of advanced heart failure, with sympathomimetic drugs that increase intracellular cAMP. The relevance of these observations to the pathophysiology of heart failure in humans is enhanced by recent data from our laboratory that implicate eNOS-derived NO in the suppression of some cardiac arrhythmias. We have found recently that adult ventricular myocytes from eNOS-/- mice were more susceptible to cardiac glycoside (ouabain)-induced afterdepolarizations than myocytes from wild-type mice (I. Kubota, X. Han, D. Opel, M.A. Arstall, T. Michel, and R.A. Kelly, manuscript under review). Ouabain-induced afterdepolarization in eNOS-/- myocytes could be suppressed by a pharmacologic NO donor, but this protective effect could be ablated by ODQ, an inhibitor of guanylyl cyclase, implicating NO-dependent generation of cGMP in mediating the anti-arrhythmic effect of eNOS activation.

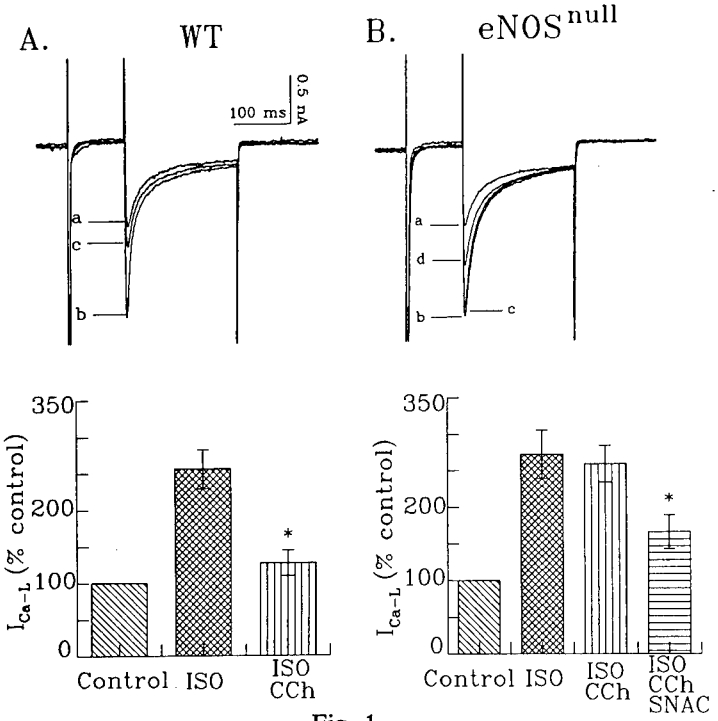

Fig. 1

The effects of isoproterenol (ISO) and carbachol (CCh) on L-type calcium current (I_{Ca-L}) in ventricular myocytes from both WT and eNOS^{null} mice are shown. **(A)** CCH significantly inhibited ISO-stimulated I_{Ca-L} in WT myocytes. Current traces labeled a, b, and c were recorded under control conditions (a), during ISO infusion (10 nM) (b), and during CCh infusion (10 μM) in the continuous presence of ISO (c) (n= 21 cells). **(B)** CCh had no significant effect on ISO-stimulated I_{Ca-L} in ventricular myocytes from eNOS^{null} mice. Current traces were recorded under control conditions (a), during ISO infusion (b), during CCH infusion in the continuous presence of ISO (c), and during infusion of the NO donor S-nitroso-acetyl-cysteine (SNAC) (100 μM) in the presence of ISO and CCh. Note that direct application of NO caused a significant inhibition in ISO-stimulated I_{Ca-L} (d). (From [19]; Copyright 1998; National Academy of Sciences, U.S.A.).

Much of the controversy in the literature to date regarding whether M2AChR agonists control cardiac myocyte contractile function has been due to the use of pharmacologic NO donors, as well as a number of pharmacologic antagonists of specific components of the M2AChR-eNOS-guanylyl cyclase signaling pathway. However, pharmacologic NO donors cannot mimic the spatial and temporal constraints on NO generated endogenously within cells, such as within caveolar microdomains. We believe the data described above in eNOS-/- myocytes demonstrates conclusively that eNOS in cardiac myocytes — and likely endothelial cells as well — is activated upon agonist-initiated translocation of the M2AChR into sarcolemmal caveolae.

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CONTROL ELEMENTS OF MUSCARINIC RECEPTOR GENE EXPRESSION

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Summary

Studies describing the structures of the M_1, M_2 and M_4 muscarinic acetylcholine receptors (mAChR) genes and the genetic elements that control their expression are reviewed. In particular, we focus on the role of the neuron-restrictive silencer element/restriction element-1 (NRSE/RE-1) in the regulation of the M_4 mAChR gene. The NRSE/RE-1 was first identified as a genetic control element that prevents the expression of the SCG-10 and type II sodium channel (NaII) genes in non-neuronal cells in culture. The NRSE/RE-1 inhibits gene expression by binding the repressor /silencer protein NRSF/REST, which is present in many non-neuronal cell lines and tissues. Our studies show that although the expression of the M_4 mAChR gene is inhibited by NRSF/REST, this inhibition is not always complete. Rather, the efficiency of silencing by NRSF/REST is different in different cells. A plausible explanation for this differential silencing is that the NRSF/RE-1 interacts with distinct sets of promoter binding proteins in different types of cells. We hypothesize that modulation of NRSF/REST silencing activity by these proteins contributes to the cell-specific pattern of expression of the M_4 mAChR in neuronal and non-neuronal cells. Recent studies that suggest a more complex role for the NRSE/RE-1 in regulating gene expression are also discussed.

Key Words: **M_4 muscarinic acetylcholine receptor, gene expression, silencer element, transcription factor, neuron**

The five known subtypes of mAChR are widely expressed in neuronal and non-neuronal cells and tissues. Understanding the molecular mechanisms by which cell-specific expression of mAChR subtypes is attained should provide important insights into the development of the tissues where they are expressed. This is particularly true for the central nervous system, where the expression of mAChR subtypes is restricted to discrete populations of neurons. Thus, an understanding of the molecular mechanisms regulating mAChR gene expression should provide a window for understanding development of the brain. In addition to providing these insights, an understanding of the how the expression of each receptor gene is regulated by extracellular stimuli such as neurotransmitters, growth factors and drugs may lead to practical methods for manipulating levels of mAChR in selected populations of cells for therapeutic purposes.

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Recent work in three laboratories has lead to the molecular cloning of the M_1 (1), M_2 (2, 3) and M_4 (4, 5, 6, 7) mAChR genes and provided our first look at the molecular mechanisms by which these genes are regulated. In this paper we will briefly summarize the results of these studies, giving particular emphasis to the regulation of the M_4 mAChR gene by the NRSE/RE-1 silencer element. We will also discuss the role of the NRSE/RE-1 in the regulation of mAChR gene expression in light of recent studies that suggest that the activities of this control element may be complex.

Structures of the M_1, M_2 and M_4 mAChR genes

Structures of the rat M_1, chicken M_2 and rat M_4 mAChR genes are shown in Figure 1. In each of these genes, the coding exon, which lacks introns, is preceded by one or two non-coding exons and an intron of 13.5 to 4.4 kb. The sites of transcription initiation for each gene were determined by RNase protection, primer extension, and in the case of the M_1 mAChR gene, PCR analysis. Transcription initiation of the M_1 mAChR gene takes place at 2 closely spaced sites located 657 bp upstream from the 5'-end of the intron. By contrast, transcription of the M_2 mAChR gene is initiated at least 5 sites within a 146 bp segment located 321 bp upstream from the 5'-end of the intron. The M_4 mAChR gene has 2 two sites of transcription initiation located around 160 bp and 496 bp from the 5'-end of the first intron. A functional promoter for each gene was identified within the region containing the transcription initiation sites and flanking upstream and downstream sequences. Each of these promoters lacks TATA and CAAT boxes that are correctly positioned for transcription initiation, but contain consensus binding sites for well known transcription factors, including: Sp-1, NZF-1, AP-1, AP-2, NFκB and Oct-1 (M_1), SP-1, AP-2, GATA (M_2) and Sp-1, AP-1, AP-2, AP-3 and Zif268 (M_4) [see ref 1-7 for the sequences and detailed descriptions of each promoter.]

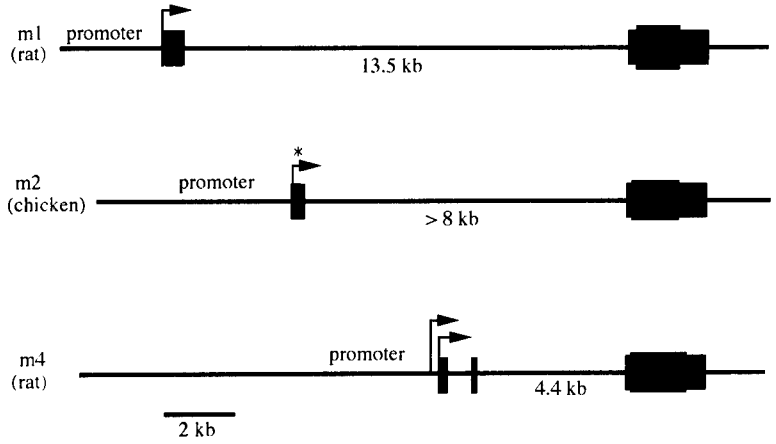

Fig. 1

Structures of the M_1, M_2 and M_4 mAChR genes as determined by restriction enzyme mapping, southern hybridization, PCR analysis and DNA sequencing of genomic DNA and cDNAs and by RNase protection and S1-nuclease studies. The asterisk "*" denotes multiple transcriptional start sites for the M_2 mAChR gene. Exons are represented by black boxes and coding regions by grey boxes. Introns and 5' and 3' flanking segments of the chromosome are represented by solid lines.

M_1 and M_2 promoters direct cell-type specific gene expression

Transient transfection assays show that DNA fragments from the upstream region of each gene can direct cell type-specific expression of reporter genes in cell lines. Thus, N. Buckley and coworkers found that a DNA fragment comprised of 0.9 kb of upstream sequences plus the 0.657 kb exon of the M_1 mAChR gene drives expression of a luciferase reporter gene in neuronal IMR32 cells, but not in 3T3 fibroblasts (1). N. Nathanson and coworkers demonstrated that a 0.789 kb segment of the M_2 upstream region directs robust expression of a luciferase reporter gene in chicken primary culture heart cells, but gives less than maximal expression in mouse SN56 septal/neuroblastoma cells (2). An expression vector containing 2 kb of upstream sequences yields approximately the same levels of expression in heart cells, but more than doubles the levels of expression in SN56 cells, suggesting that the region between 0.789 and 2 kb contains a regulatory element required for maximal expression in neuronal cells. By contrast, an expression vector containing 3 kb of upstream sequences directs lower levels of reporter gene expression in both heart and SN56 cells, suggesting the presence of a repressor/silencer element in the 2 to 3 kb upstream region.

In the same study, it was shown that an expression vector containing 2 kb of upstream sequences was induced 4-fold in SN56 cells following stimulation with ciliary neurotrophic factor (CNTF) or leukemia inhibitory factor (LIF). A more recent study (3) by the same group showed that maximal expression of the M_2 gene in heart cells requires a binding site for the transcription factor GATA within the first, non-coding exon. These authors also demonstrated that nuclear extracts prepared from embryonic heart cell cultures contain a protein that binds to this site, and that exogenously expressed GATA-4, -5, and -6 isoforms transactivated the M_2 promoter in the human choriocarcinoma cell line JEG-3, which normally expresses only very low levels of mAChR. GATA -4, -5 and -6 are "zinc finger"-containing transcription factors previously shown to regulate lineage-specific genes in mesoderm-derived tissues, including heart (8, 9, 10, 11). These GATA isoforms are the first transcription factors to be shown to positively regulate cell-type specific expression of a mAChR gene.

Regulation of M_4 mAChR gene expression by NRSE/RE-1 in non-neuronal cells

Studies carried out by our group (6, 7) and independently by N. Buckley and coworkers (4, 5) have shown that expression of the M_4 mAChR gene is repressed in non-neuronal cells in culture by a NRSE/RE-1 silencer element located in the 5' upstream region of the M_4 mAChR gene. The M_4 NRSE/RE-1 is similar in sequence to the NRSE/RE-1 element first identified in the promoters of the SCG-10 (12) and type II sodium channel (NaII) genes (13) and shown to restrict the expression of these genes in non-neuronal cell lines. The M_4 NRSE/RE-1 is located 837 bp upstream from the site of transcription initiation identified in our studies and 500 bp upstream from the transcription initiation site identified by Buckley and coworkers. The orientation of the m4 NRSE/RE-1 with respect to the direction of transcription is opposite to that of NRSE/RE-1 elements in the SCG-10 and NaII promoters. Sequences similar to the SCG-10 and NAII NRSE/RE-1 have been identified in the regulatory regions of more than 40 gene expressed in neurons, as well as in the promoters of some genes that are expressed in non-neuronal cells (14. 15). A comparison of the sequence of the M_4 NRSE/RE-1 with an experimentally determined consensus NRSE/RE-1 sequence (14) is shown in Figure 2.

Transient transfection assays using expression plasmids containing various segments of the M_4 regulatory region show that the M_4 NRSE/RE-1 represses the expression of luciferase reporter genes in myoblast-derived L6 cells and fibroblast-like 3Y1B, 3T3 and CHO cells (4-7). As is

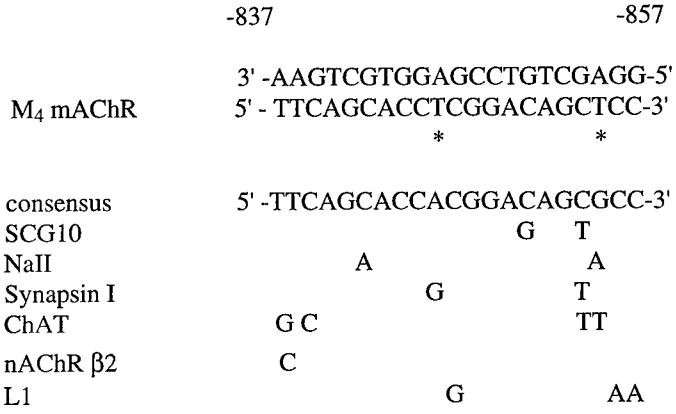

Fig. 2

Comparison of the M_4 NRSE/RE-1 with the experimentally determined NRSE/RE-1 consensus sequence and selected NRSE/RE-1 that have been shown to repress gene expression in non-neuronal cells. See (14) for references.

common for enhancer/silencer elements, the M_4 NRSE/RE-1 continues to be functional when it is displaced from its normal position within the regulatory region well as when its orientation is reversed (5, 7). Nuclear extracts of L6 cells (7) and 3T3 and CHO cells (5) contain proteins that specifically bind the M_4 NRSE/RE1 in gel mobility shift assays, suggesting that the M_4 NRSE /RE-1 inhibits gene expression in those cells by binding a silencer/repressor protein. Consistent with this model, nuclear extracts of the neuronal cell lines PC12, PC12D, and NG108-15 were shown to lack NRSE/RE-1 binding activities (5,7).

The NRSE/R1 inhibits expression of the SCG-10 and NaII genes in non-neuronal cell lines by binding the silencer/repressor protein NRSF/REST (16, 17) NRSF/REST is a large protein containing 1114 amino acids (human) that can be divided into several structural domains including: the amino-terminal region, a segment containing 8 Cys_2His_2-type "zinc fingers," a basic amino acid-rich region, a nuclear translocation signal, a novel proline-rich repeated motif (6 reiterations) and a carboxyterminal region containing a solitary zinc finger (15-18).

To determine whether the M_4 NRSE/RE-1 inhibits gene expression by binding NRSF/REST, we examined the effects of exogenously produced NRSF/REST on gene expression from a reporter plasmid containing the M_4 NRSE/RE-1 linked to the M_4 proximal promoter region (7). As a control, we also examined the effect of exogenously produced NRSF/REST on gene expression from a construct containing the NaII NRSE/RE-1 linked to a segment of the NaII proximal promoter. These experiments were carried out in NG108-15 cells, which do not produce endogenous NRSF/REST. The results showed that exogenously produced NRSF/REST completely blocked expression from the NaII expression vector, but only partially blocked the expression from the M_4 expression vector. To determine if the efficiency of silencing was determined by the origin of the NRSE/RE-1 or the proximal promoter, we repeated the above experiments using expression plasmids containing chimeric promoters, i.e, with the M_4 NRSE /RE-1 linked to the NaII proximal promoter or the NaII NRSE/RE-1 linked to the M_4 proximal promoter. These experiments showed that the M_4 NRSE/RE-1 nearly completely inhibited expression from the NaII proximal promoter, but the NaII NRSE/RE-1 only partially blocked

expression from the M_4 proximal promoter. Thus, partial inhibition is a property of the promoter, rather than the NRSE/RE-1.

In contrast to the partial inhibition of M_4 promoter activity in NG108-15 cells expressing exogenous NRSF/REST, expression from both M_4 and NaII reporter plasmids was efficiently blocked in L6 cells. This result shows that efficiency of silencing of the NRSE/RE-1 within the M_4 promoter depends upon the type of cell in which it is located. These considerations prompted us to examine whether the proteins that bound to the minimal M_4 promoter are different in different cells. Mobility shift analysis of complexes formed between a ^{32}P-labeled, 90 bp segment of the M_4 minimal promoter and proteins in nuclear extracts prepared from NG108-15 and L6 cells revealed differences in the complexes formed by proteins in the two extracts. This result suggests that different sets of proteins bind the M_4 minimal promoter in different cells. An attractive hypothesis is that the efficiency of NRSE/RE-1 silencing is determined by how NRSF/REST interacts with these promoter-binding proteins.

Regardless of the exact mechanism, the above observations indicate that the silencing activity of the NRSE/RE-1 is not absolute, but rather can be modulated, possibly to allow exceptions to the "neuron-specific" expression of the genes it regulates. A loosening of the silencing activity of this control element could, for example, explain the reported expression of M_4 mAChR mRNA in non-neuronal tissues, including developing chicken heart (19), rabbit lung (20), bovine adrenal glands (21) and human blood mononuclear cells (22). A model for the regulation of the NaII and M_4 mAChR gene expression by the NRSE/RE-1 and NRSF/REST is shown in Figure 3.

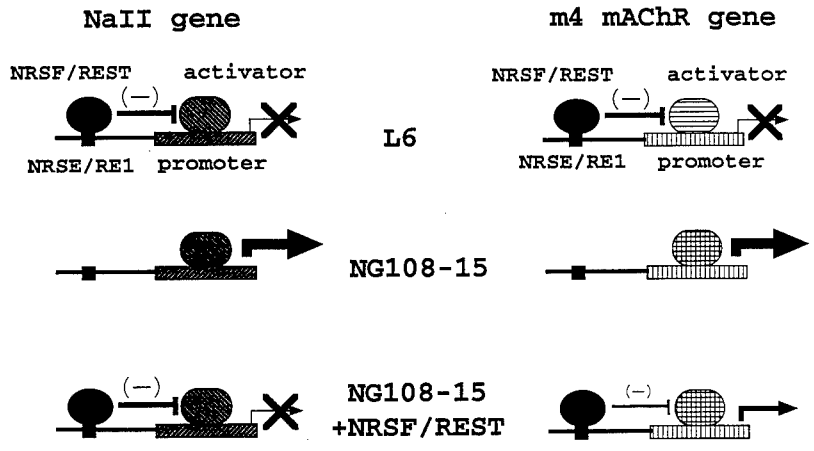

Fig. 3

(Top) Expression from the NaII and M_4 promoters is efficiently blocked in L6 cells, which produce endogenous NRSE/REST. (Middle) NaII and M_4 promoters are active in NG108-15 cells, which lack NRSF/REST. (Bottom) Expression from the NaII promoter is efficiently blocked by exogenously produced NRSE/REST in NG108-15 cells, but expression from the M_4 promoter is only partially blocked. The difference in efficiency of silencing of the M_4 promoter in L6 and NRSF/REST-expressing NG108-15 cells may be due to differences in the proteins that bind the minimal promoter in each cell.

Regulation of M_4 mAChR gene expression by NRSE/RE-1 in neurons

A recent study by T. Timmusk and coworkers (23) found that contrary to earlier reports, NRSF/REST is, in fact, expressed in neurons in the brain. The pattern of expression determined by in situ hybridization was shown to be non-uniform, with highest levels of expression in neurons of the hippocampus, pons/medulla and midbrain and low or undetectable levels in the caudate putamen, globus pallidus and nucleus accumbens. As pointed out by these authors there is an apparent inverse correlation between the expression of M_4 mRNA and NRSE/REST. For example, M_4 mRNA is expressed at relatively high levels in the caudate putamen (24, 25) and in the septum and basal forebrain (26, 27) where NRSE/REST levels are low (23). Thus, the cell-specific expression of the M_4 mAChR in neurons of the brain may also be determined by the silencing activity of NRSF/REST. Furthermore, the ability to modulate the silencing activity of NRSF/REST by changing the proteins that interact with the minimal promoter could provide yet another mechanisms by which M_4 AChR could be expressed in specific populations of cells.

The data discussed up to this point leaves us with a very simple model to explain the regulation of the M_4 mAChR gene: the M_4 mAChR is expressed as a "default" choice in cells that do not express NRSF/REST, while expression is prevented in most non-neuronal cells and some neurons where NRSF/REST is expressed. To explain the exceptional cases where the M_4 mAChR is expressed in tissues that produce NRSF/REST, we invoke the existence of promoter-binding protein that weaken the silencing activity of NRSF/REST. Although this simple model can account for many of the experimental observations to date, there are already clear indications that the real situation must be more complex.

For example, two recent studies that examine the effects of mutating or deleting the NRSE/RE-1 on promoter activity in transgenic mice, suggest that the NRSE/RE-1 can also function as a positive modulator of gene expression. Thus, J.-P. Changeux and coworkers (28, 29) studying the nicotinic acetylcholine receptor (nAChR) β2 subunit promoter in transgenic mice, found that mutation of the NRSE/RE1 in the 5' untranslated region of the nAChR β2 gene causes the loss of β-galactosidase reporter gene expression in most neurons where the wild-type promoter drives expression. In addition, transfection assays using SK-N-Be neuroblastoma cells showed that the NRSE/RE-1 stimulates reporter gene expression when it is located near the site of transcription initiation in expression constructs. This stimulation of transcription by NRSE/RE-1 apparently depends upon small amounts of NRSF/REST produced by these cells since coexpression of NRSE/REST antisense RNA blocks the expression. Likewise, F. Jones and coworkers (30, 31) showed that deletion of the NRSE/RE1 located in the second intron of the L1 adhesion molecule gene causes loss of L1 promoter activity in many neurons during postnatal development and in the adult. The inference that can be drawn from these studies is that normal expression of some genes in neurons depends upon having a functional NRSE/RE-1.

It is also worth noting that although both studies report some ectopic reporter gene expression in mice harboring expression vectors with defective NRSE/RE-1, the inactivation of this element did not result in the widespread expression of β-galactosidase in glial cells. Furthermore, in situ hybridization analysis detected NRSF/REST primarily in neurons and not glial cells (23). Thus, the lack of expression of M_4 mAChR and many other neuron specific genes in glia probably depends upon mechanisms distinct from silencing by NRSE/RE-1. Finally, the discovery by J. Strominger and coworkers (32) that NRSF/REST (designated XBR) is expressed at high levels in the "lymphatic compartment," including spleen, thymus and peripheral blood leukocytes, where it

represses transcription of a major histocompatibility complex class II gene, suggests that the functions of NRSF/REST extend beyond mediating neuronal/non-neuronal gene expression. In light of these recent studies, it is obvious that much work remains to be done to establish the exact role of the NRSE/RE-1 and NRSF/REST in the regulation of the M_4 mAChR gene.

Future directions

The molecular cloning of the regulatory regions of the mAChR genes and the identification of several control elements promise to open up many new avenues of research concerning these important receptors. For example, having the promoter regions in hand will facilitate the construction of chimeric mice where reporter genes such as β–galactosidase or green fluorescent protein are placed under the control of the endogenous mAChR promoters for use in visualizing mAChR gene expression at the single cell level during development. Such studies should yield valuable hints concerning the functions of the various subtypes mAChR and important insights into the development of complex organs, such as the brain. The cloned mAChR promoters may also find practical applications, such as use in the rapid screening protocols for drugs that modulate levels of mAChR gene expression. Efforts to understand the molecular mechanisms that control mAChR gene expression are of course now only in their initial stages, and practical applications probably lie even further down the road. The availability of cloned mAChR promoters, however, should provide investigators with good points of departure and powerful tools for their future work.

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ENDOCYTOSIS AND RECYCLING OF MUSCARINIC RECEPTORS

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Summary

Agonist stimulation causes the endocytosis of many G protein-coupled receptors, including muscarinic acetylcholine receptors. In this study we have investigated the agonist-triggered trafficking of the M_3 muscarinic receptor expressed in SH-SY5Y human neuroblastoma cells. We have compared the ability of a series of agonists to generate the second messenger Ins(1,4,5)P_3 with their ability to stimulate receptor endocytosis. We show that there is a good correlation between the intrinsic activity of the agonists and their ability to increase the rate constant for receptor endocytosis. Furthermore, on the basis of our results, we predict that even very weak partial agonists should under some circumstances be able to cause substantial receptor internalization. Receptor endocytosis occurs too slowly to account for the rapid desensitization of the Ca^{2+} response to carbachol. Instead, receptor endocytosis and recycling appear to play an important role in resensitization. After an initial agonist challenge, the response to carbachol is fully recovered when only about half of the receptors have been recycled to the cell surface, suggesting that there is a receptor reserve of about 50%. Removal of this reserve by receptor alkylation significantly reduces the extent of resensitization. Resensitization is also reduced by inhibitors of either endocytosis alone (concanavalin A) or of endocytosis and recycling (nigericin). Finally, the protein phosphatase inhibitor calyculin A also reduces resensitization, possibly by blocking the dephosphorylation of the receptors in an endosomal compartment.

Key Words: muscarinic receptors, endocytosis, recycling, desensitization

Newly-synthesized G protein-coupled receptors are synthesized on ribosomes bound to the membrane of the rough endoplasmic reticulum. They are then transported via the Golgi complex to the plasma membrane. This is where the bulk of the receptors reside in unstimulated cells. Agonist stimulation usually causes efficient and extensive receptor endocytosis. The receptors are delivered to intracellular compartments (endosomes) from which they may be either recycled to the plasma membrane or transported on to lysosomes for degradation (1, 2). Two major unresolved questions are: how does agonist stimulation trigger receptor endocytosis, and what is the functional significance of this agonist-induced receptor trafficking? The best-characterized receptor from this point of view is the β_2-adrenoceptor. Agonist stimulation of this receptor causes phosphorylation at different intracellular sites by protein kinase A and β-adrenoceptor kinase, or G protein-coupled receptor kinase 2 (GRK2, Ref. 3). Phosphorylation by GRK2, on the carboxy-terminal tail of the receptor, causes the recruitment of the cytosolic protein β-arrestin, which binds to the third intracellular loop and carboxy-tail of the receptor and uncouples it from the G protein, Gs (4). These phosphorylation events are critically involved in receptor desensitization, which is seen as a reduction in the ability of the receptor to generate second messenger in the continued presence of agonist (3). β-arrestin is also involved in agonist-triggered endocytosis of the β_2-adrenoceptor (5), linking the receptor to clathrin and thereby recruiting the receptor into coated pits (6). The development of desensitization is more rapid than the endocytosis of the receptors, suggesting that

endocytosis is unlikely to contribute significantly to desensitization. Receptor endocytosis delivers the receptor into an endosomal compartment, where a low luminal pH supports an enhanced phosphatase activity (7). The receptor is efficiently dephosphorylated and then recycled to the plasma membrane, where it is once again able to respond to extracellular agonists. The purpose of endocytosis and recycling of the β_2-adrenoceptor therefore appears to be to facilitate resensitization. In support of this idea, inhibition of endocytosis by concanavalin A or hyperosmolar sucrose, of recycling by monensin or of dephosphorylation by calyculin A all inhibit receptor resensitization (8). Furthermore, overexpression of arrestin stimulates both internalization and resensitization of the β_2-adrenoceptor (9).

A major challenge now is to determine how much of what is known about the trafficking of the β_2-adrenoceptor is applicable to other G protein-coupled receptors. In the case of the muscarinic acetylcholine receptors, there is clear evidence that the receptors are substrates for GRKs, both *in vitro* (10, 11) and *in vivo* (12). However, the role of GRKs in mediating agonist-triggered receptor endocytosis, and the role of receptor endocytosis/recycling in desensitization/resensitization are still rather unclear, and conflicting results have been reported (11, 12). Furthermore, other kinases have been reported to phosphorylate msucarinic receptors in an agonist-dependent manner (13). Like the β_2-adrenoceptor, the M_2 muscarinic receptor has been shown to bind β-arrestin *in vitro* in an agonist- and phosphorylation-dependent manner (14). However, when expressed in HEK-tsA201 cells, arrestin appeared to be required for desensitization but not for phosphorylation-dependent internalization (15). Some of this confusion appears to arise from the use of various cell lines transfected with particular receptors. The behaviour of muscarinic receptors in different cell types is known to vary, and even in a single cell type, the results obtained may depend on the receptor subtype being studied. For instance, M_3 receptors endogenously expressed in SH-SY5Y cells and M_4 receptors in NG108-15 cells are both endocytosed efficiently in response to agonist stimulation. However, in transfected CHO cells, M_2 and M_4 receptors (which couple to G_i and inhibit adenylate cyclase) are endocytosed efficiently whereas M_1 and M_3 receptors (which couple to $G_{q/11}$ and stimulate phospholipase C) are not (16). In addition, evidence has been presented recently (15, 17) that the M_2 subtype, in contrast to the other subtypes, is internalized via a dynamin-independent pathway. The clear implication is that the different subtypes of muscarinic receptors have different internalization mechanisms, and that a complete internalization machinery for a particular subtype might not operate in a 'foreign' cell. For this reason, we have studied the mechanisms and functional consequences of receptor endocytosis and recycling in cell lines that naturally express a particular receptor subtype. In this paper, we focus on the SH-SY5Y human neuroblastoma cell line, which expresses predominantly M_3 muscarinic receptors (18). We address two major issues: the relationship between agonist intrinsic activity and receptor endocytosis, and the role of receptor trafficking in the processes of desensitization and resensitization.

Methods

The methods used in these studies have been described in detail elsewhere (19, 20). Experiments were performed on SH-SY5Y cells growing on 24-well culture plates. The mass of Ins(1,4,5)P_3 generated in response to agonist stimulation was measured in cell extracts through the displacement of [^{3}H]Ins(1,4,5)P_3 from an Ins(1,4,5)P_3 binding protein. Agonist-stimulated internalization of muscarinic receptors was measured via the reduction in the binding of the membrane-impermeant radioligand [^{3}H]*N*-methylscopolamine ([^{3}H]NMS). To measure receptor recycling to the plasma membrane, internalization was first allowed to reach steady state. In some experiments, the cells were then cooled to prevent further receptor trafficking, and receptors remaining at the cell surface were alkylated using propyl-benzilylcholine mustard (PrBCM). Recycling was quantitated through the increase in [^{3}H]NMS binding after the cells were warmed to 37°C. For population $[Ca^{2+}]_i$ measurements, cells growing on glass coverslips were loaded with fura-2 by incubation in a solution containing fura-2 acetoxymethylester. Fura-2 fluorescence was measured using a Hitachi F-2000 fluorescence spectrometer (excitation wavelengths 340 nm and 380 nm; emission wavelength 510 nm). Fluorescence ratios were converted to free $[Ca^{2+}]$ by reference to a look-up table created using Ca^{2+} standards.

Receptor trafficking was fitted to a two-compartment model, which assumes that receptors cycle between the cell surface and endosomes. The number of receptors recycled to the cell surface

at any time, R_s, is given by

$$R_s = R_{e0}\left(1 - e^{-k_r t}\right)$$

where R_{e0} is the number of receptors in endosomes at t=0, and k_r is the rate constant for recycling. Hence k_r can be determined from a plot of R_s against t.

The number of receptors remaining at the cell surface after internalization has been allowed to proceed to steady state may be written as

$$R_{s,ss} = \frac{k_r\left(R_{s0} + R_{e0}\right)}{k_r + k_e}$$

where R_{s0} and R_{e0} are the number of receptors initially at the cell surface and in endosomes, respectively, and k_r and k_e are the rate constants for recycling and endocytosis, respectively. k_e can therefore be calculated, assuming that k_r is known.

Results and Discussion

Relationship between agonist intrinsic activity and the rate of receptor endocytosis

Normally, agonists, but not antagonists, trigger receptor internalization, suggesting that it is the activated form of the receptor, that couples to G proteins, which is also responsible for initiating receptor endocytosis. Studies of the relationship between agonist activity and their ability to trigger receptor internalization have, however, generated confusing results. For example, it has been shown that enkephalins and etorphine stimulate endocytosis of μ- and δ-opioid receptors in transfected cells and enteric neurons (21, 22), but the full agonist morphine does not. These results suggest that the opioid receptors are able to adopt multiple activated conformations. Furthermore, there is evidence that antagonists as well as agonists might be able to trigger receptor internalization. For instance, it has recently been reported that an antagonist at the cholecystokinin receptor can stimulate internalization (23). With these results in mind, we compared the ability of a series of muscarinic ligands acting at M_3 muscarinic receptors in SH-SY5Y cells to generate the second messenger Ins(1,3,4)P_3 and to modify receptor trafficking. Our aim was to determine whether the same receptor conformation was responsible for activating both second messenger generation and receptor endocytosis.

Addition of muscarinic agonists to SH-SY5Y cells caused a biphasic Ins(1,4,5)P_3 response, with a rapid increase that reached a peak at 10 sec, followed by a reduction over the next 120 sec to a sustained plateau phase. Dose-response curves for the peak Ins(1,4,5)P_3 responses were constructed for eight muscarinic ligands. It was found that carbachol, oxotremorine-M and methacholine were full agonists, whereas bethanechol, arecoline and pilocarpine were partial agonists, causing 52%, 26% and 8%, respectively, of the maximal response to carbachol (Table I). *N*-methylatropine and McN-A-343 were without effect (not included in the Table). The rank order of potency for stimulation of Ins(1,4,5)P_3 production was: oxotremorine-M>methacholine> carbachol≈arecoline≈pilocarpine >>bethanechol.

To measure receptor internalization in response to stimulation, cells were treated with ligand for 30 min, to allow receptor cycling between the plasma membrane and intracellular compartments to reach steady state, and then the number of receptors remaining at the cell surface was measured through the binding of [^{3}H]NMS. The full agonists carbachol, methacholine and oxotremorine-M all caused maximal (about 90%) receptor internalization. The partial agonists bethanechol, arecoline and pilocarpine caused the internalization of 82%, 78% and 19% of surface receptors at steady state. *N*-methylatropine and McN-A-343, which did not cause detectable Ins(1,4,5)P_3 production, did not affect receptor distribution. Recycling of receptors to the cell surface following agonist-triggered internalization followed an exponential time-course, and the rate constant for recycling (approx. 0.055 min^{-1}) did not depend upon which agonist had induced internalization.

TABLE I

Comparison of the Ability of Ligands to Stimulation Ins(1,4,5)P_3 Production, Receptor Internalization and the Endocytotic Rate Constant

Compound	Ins(1,4,5)P_3 Response		Receptor Internalization		Endocytotic Rate Constant	
	R_{max}	EC_{50}	I_{max}	EC_{50}	$k_{e(max)}$	EC_{50}
	%	μM	%	μM	min^{-1}	μM
Carbachol	100	74	90	3.5	0.54	24
Oxotremorine-M	97	6.6	90	0.75	0.55	6.7
Methacholine	94	39	89	4.8	0.49	40
Bethanechol	52	1200	82	130	0.26	552
Arecoline	26	150	78	6.9	0.18	38
Pilocarpine	8.4	130	19	57	0.01	68

Data from Ref. 19.

Although the rank order of agonist activity for Ins(1,4,5)P_3 production and receptor internalization is the same (oxotremorine-M>methacholine>carbachol≈arecoline≈pilocarpine >>bethanechol), the ability of the three partial agonists to cause receptor internalization is greater than one would expect from their ability to generate Ins(1,4,5)P_3. We have shown previously (1, 2) that the extent of internalization is a function of both endocytotic and recycling rate constants. When the internalization data were used to calculate values for the maximal endocytotic rate constant $k_{e(max)}$, these values were found to correlate very well with the ability of the agonists to generate Ins(1,4,5)P_3. The agonist potencies for the two effects are also of the same order. This result is consistent with the generally accepted view that muscarinic agonists interact with a conserved binding site (24, 25), and indicates that the same activated conformation of the receptor is responsible for activating both second messenger generation and receptor endocytosis.

The extent of receptor internalization in response to agonist stimulation depends not only on k_e, but also on k_r and the maximum possible rate constant for endocytosis $k_{e(max)}$, factors which are cell-specific. Mathematical modelling (19) can be used to show that when k_r is small and $k_{e(max)}$ is large, as in the SH-SY5Y cell line used here, even a very weak partial agonist, with an intrinsic activity of less than 5% of that of a full agonist, will cause substantial (20% or greater) receptor internalization. The activity of such a compound might be difficult to detect, and it might consequently be classified as an antagonist, generating confusion about the requirements for the triggering of receptor internalization.

Role of receptor endocytosis and recycling in resensitization

Carbachol caused a biphasic elevation of [Ca^{2+}] in SH-SY5Y cells, consisting of an initial peak at 5-10 sec followed by a sustained plateau. Pre-exposure of cells to a saturating concentration of carbachol (1 mM) caused a reduction in the peak response to a second challenge with carbachol to 29% of the initial response. The half-time of the desensitization was about 30 sec. In contrast, receptor internalization in response to carbachol stimulation occurred with a half-time of approximately 4 min, indicating that internalization is not likely to play a major role in desensitization. The time-course of resensitization of the response to carbachol, following removal of a 5-min desensitizing dose and a 3-min wash period, is shown in Fig. 1. Resensitization was approximately half-complete after 6 min of recovery, and complete after 9 min. After a 5-min treatment with carbachol, surface receptor number had decreased to 33% of control values. After

removal of the agonist, recycling of receptors to the plasma membrane occurred with a half-time of about 14 min. Maximal recovery occurred after about 45 min and amounted to only 65% of the original number of receptors at the cell surface. This incomplete recovery was not due to receptor degradation, because the binding of the membrane-permeant radioligand [^{3}H]scopolamine was unaffected. The most likely explanation is that some of the internalized receptors had moved into an endosomal compartment which recycled only slowly or not at all.

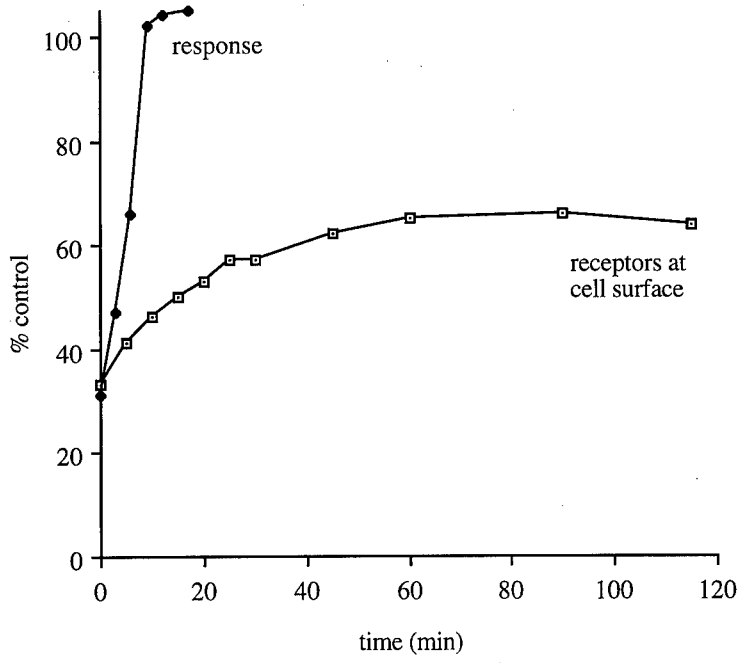

Fig. 1
Comparison of the time-courses of resensitization of the Ca^{2+} response and recycling of receptors to the cell surface. Note that the Ca^{2+} response has recovered completely when the number of receptors at the cell surface is only about 50% of control. Data from Ref. 20.

Inspection of Fig. 1 reveals that at the 9-min time point, when the response to carbachol has fully resensitized, there are only about half of the original number of receptors at the cell surface. This result suggests that SH-SY5Y cells have a receptor reserve of about 50% for the Ca^{2+} response to carbachol. To demonstrate this directly, we treated cells with the alkylating reagent PrBCM for various times. As shown in Fig. 2, this caused a progressive reduction in [^{3}H]NMS binding. However, in the same series of experiments, there was a lag of about 5 min before a signficant reduction in the peak Ca^{2+} response to carbachol was seen. A 5-min treatment with PrBCM resulted in the alkylation of about 50% of the surface receptors, confirming our prediction of a receptor reserve of 50%. In order to test the effect of this reserve on desensitization and resensitization of the response to carbachol, we compared the desensitization/resensitization time-course for cells that had been treated with PrBCM for 5 min with the time-course for untreated cells. We found that PrBCM treatment did not significantly affect the time-course or the extent of desensitization, but did reduce the extent of resensitization by about 50%. We conclude that the receptor reserve in these cells does not have a significant impact on the process of desensitization, but does contribute significantly to resensitization. Without this reserve, insufficient receptors return to the cell surface, at least over the first few minutes, to allow full recovery of the response.

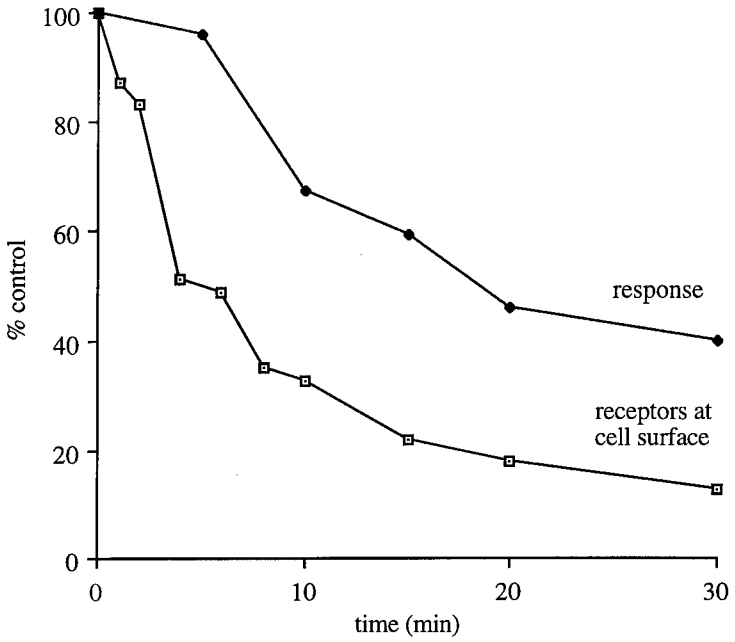

Fig. 2
Comparison of the time-courses of alkylation of receptors by PrBCM and reduction in the Ca^{2+} response to carbachol (1 mM). Note that after 5 min of PrBCM treatment the response is not substantially affected, but the number of receptors at the cell surface is reduced by about 50%. Data from Ref. 20.

To examine further the functional roles of receptor endocytosis and recycling, we tested the effects of agents that have been reported to block these processes on desensitization and resensitization of the Ca^{2+} response to carbachol. The lectin concanavalin A (con A) has been shown to block endocytosis of the β_2-adrenoceptor (8). We found that con A did not completely block endocytosis of muscarinic receptors in SH-SY5Y cells, but did reduce the rate constant for endocytosis, k_e, by about 50% (from 0.17 min^{-1} to 0.09 min^{-1}). In contrast, it had no significant effect on either the rate or the extent of receptor recycling. Con A had no effect on the extent of desensitization produced by carbachol, but did reduce the rate and extent of resensitization. For example, during a 12-min recovery period in untreated cells, the response rose from 31% to 102% of control, whereas in con A-treated cells, the response rose from 27% to 62% of control. Carboxylic ionophores, such as monensin and nigericin, have been reported to inhibit receptor recycling by interfering with the ionic balance across the membranes of endosomes (8). We found that nigericin did not affect the rate constant for muscarinic receptor recycling, k_r, but did reduce the extent of recycling - the plateau recovery of [^{3}H]NMS binding after carbachol stimulation and PrBCM treatment was 25% of control in nigericin-treated cells, compared with 41% in untreated cells. Nigericin also reduced the rate constant of endocytosis, k_e (from 0.17 min^{-1} to 0.12 min^{-1}). Nigericin had a very similar functional effect to that of con A: the extent of desensitization was unaffected, but the rate and extent of resensitization were reduced - over a 12-min recovery period, the response rose from 22% to 63%. Hence, the endocytosis and recycling of muscarinic receptors are involved in resensitization of the response, and not desensitization.

One important consequence of endocytosis of the β_2-adrenoceptor is the dephosphorylation of the receptor in an endosomal compartment. Unfortunately, the level of expression of M_3 muscarinic receptors in SH-SY5Y cells is too low to permit a direct study of the phosphorylation state of the receptor under various conditions. We did, however, look for indirect evidence that

receptor dephosphorylation might be involved in desensitization/resensitization by testing the functional effect of the serine/threonine phosphatase inhibitor calyculin A. We found that calyculin A increased the extent of desensitization - the carbachol response was reduced to 12% of control in calyculin-treated cells, compared with 31% in untreated cells. It also reduced the extent of resensitization - over a 12-min recovery period, the response rose to 49% of control.

Our results suggest that agonist-stimulated trafficking of M_3 receptors in SH-SY5Y cells contributes to receptor resensitization, perhaps through an enhancement of receptor dephosphorylation by an intracellular phosphatase. This scenario is similar to that for the archetypal G protein-coupled receptor, the β_2-adrenoceptor, and very different from that for the M_4 muscarinic receptor, where internalization has been reported to delay receptor resensitization (26). It would appear, then, that there are no general rules governing the relationship between receptor trafficking and receptor desensitization/resensitization, even within the small family of muscarinic receptors.

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REPRESSION AND ACTIVATION OF MUSCARINIC RECEPTOR GENES

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Summary

The specific cellular response to muscarinic receptor activation is dependent upon appropriate expression of each of the five muscarinic receptor genes by individual cells. Here we summarise recent work describing some of the genomic regulatory elements and transcriptional mechanisms that control expression of the M_1 and M_4 genes.

Key Words: muscarinic receptor genes, transcription, promoter, activator, repressor

Muscarinic receptors are distributed widely throughout the mammalian central and autonomic nervous systems where each of the five muscarinic receptor genes has a unique expression profile (1-3). We have focused our attention on two of these genes, the M_1 and M_4. Expression patterns of the M_1 and M_4 genes are broadly similar: both are expressed by autonomic ganglia (2, 3) and, in the central nervous system, expression is largely restricted to telencephalic regions (1, 4, 5). In the latter case, many neurons of the cortex, striatum and hippocampus express both genes, but other neuronal populations express only one or neither resulting in a matrix of cells expressing, both, one or neither gene. These two genes therefore present a model with which to examine the DNA/protein and protein/protein interactions that give rise to differential patterns of expression of individual muscarinic receptor genes. In the following account, we present an overview of the gene structure and genomic regulatory elements of both of these genes and a dissection of one transcriptional mechanism that may be responsible for silencing M_4 expression in some non-expressing cells.

A. Structure of the rat M_1 and M_4 genes

All five mammalian muscrinic receptor genes (6,7) share one common feature with several other members of the G-protein receptor gene superfamily - their open reading frames are contained within a single exon. The rat M_1 gene contains at least one non-coding exon 13.5kb upstream of the coding exon (8). Comparison with porcine cDNA indicates a region of homology further upstream but we can find no evidence that this region is transcribed in rat brain. In addition we have identified an alternative downstream exon that is transcribed in mouse tissue but not in rat or human tissues so far examined. Such variations could represent either tissue or species specific splicing. Like the M_1 gene, the rat M_4 gene also contains upstream exon(s). Two studies have shown that transcription may be initiated from two sites separated by about 300bp (9,10). We present evidence that these two transcriptional start sites may be differentially regulated (see later).

B. Transcriptional control of M_4 gene expression

Transcriptional mapping of the M_4 promoter has identified two transcription initiation sites (upstream t.i.s. and downstream t.i.s.) both of which appear to be constitutively active (11,12). Examination of the 5'flanking region revealed a RE1/NRSE (Repressor Element 1 / Neuron Restrictive Silencer Element). RE1/NRSE elements were originally identified in the SCG10 (11) and NaII (12) genes and have been subsequently identified in over thirty genes most of which are neurally restricted (13). The RE1/NRSE recruits a Zn finger repressor, REST/NRSF (RE1 Silencing Transcription Factor (14) / Neuron Restrictive Silencer Factor (15)), to silence gene expression in non-neuronal cells. Using reporter constructs that employ only the upstream start site, we found that deletion of the RE1/NRSE led to expression of reporter constructs in non-expressing 3T3 and CHO cells (16). Furthermore direct juxtaposition of the RE1/NRSE to a minimal promoter construct silenced expression. Interestingly, another study used exogenous REST/NRSF and reporters containing *both* start sites to show that REST/NRSF could only partly repress reporter gene activity in NG108-15 cells whereas expression of the RE1/NRSE bearing NaII promoter in the same cells was completely repressed. Expression of both reporters was silenced in L6 cells (17). By using a series or reporters containing mutated start sites and by overexpressing RE1/NRSE sites to titrate out the effects of endogenous REST/NRSF in 3T3 cells, we have shown that only the upstream promoter is silenced by REST/NRSF while the downstream site remains refractory to REST/NRSF. Hence, it would appear that a likely resolution to this issue is that both promoters are used in cells such as NG108-15 and 3T3 resulting in partial silencing of reporter constructs containing both start sites by REST/NRSF whilst only the upstream promoter is used in cells such as L6 resulting in total silencing by REST/NRSF. Further experiments are underway to directly test this hypothesis. Hence, one component of cell specificity may be attributable to the use of alternative transcription start sites. An outstanding unanswered question then naturally arises as to how the downstream start site is silenced in non-expressing cells such as 3T3? One caveat that should be borne in mind is that all functional assays conducted so far have been carried out on transiently transfected reporter plasmids and the relevance of these silencing mechanisms needs to be assessed on chromatin not naked (or semi-naked) DNA. This is especially pertinent since silencing may well involve histone deacetylation (see later).

Although REST/NRSF was initially conceived as operating solely as a repressor in non-neuronal cells more recent work has indicated the presence of full length and truncated REST/NRSF in neuronal cells (17,18). One study has shown that mutation of the RE1/NRSE in the ß2 nicotinic receptor gene leads to silencing of gene expression in the embryonic CNS and widespread expression of the reporter gene throughout the adult CNS (20) indicating that REST/NRSF may also act as either a repressor or enhancer in some neurons. Hence, REST/NRSF may play a wider role than originally postulated and may direct M_4 expression in both non-neuronal *and* neuronal cells.

C. Transcriptional control of M_1 gene expression

Transcription control of the rat M_1 gene differs markedly from the M_4 gene. Firstly, the M_1 promoter does not appear to be constitutively active and secondly, expression appears to be controlled, at least in part, by activator elements within the first exon (8). There are no RE1/NRSE elements within the proximity of the M_1 promoter and Southern analysis indicates there are none within at least 20kb of the promoter. Reporter constructs containing as little as 372bp of 5'flanking sequence and the first exon drive cell specific expression in IMR32 neuroblastoma, a cell line that expresses the M_1 receptor. Deletion of 200bp of the first exon leads to a complete loss of expression in IMR32. Only a preliminary analysis of the DNA/protein

interactions has been carried out but it appears that there are several enhancer elements in the first exon, some of which bind ssDNA binding proteins. As yet, these proteins have not been fully characterised. These initial studies would support the notion that, despite significant similarities in the expression patterns of the M_1 and M_4 genes, distinct transcriptional mechanisms are responsible for regulating cell specific expression of these genes.

D. Repression of the M_4 promoter by the REST/NRSF silencer

A tenet of contemporary molecular neurobiology is that for the expression of thousands of genes to be restricted to specific subsets of neurons by a relatively discrete number of transcription factors requires combinatorial mechanisms i.e. no single transcription factor is likely to uniquely control the expression of one gene. In other words, REST/NRSF silencing of M_4 expression is undoubtedly one mechanism by which its expression is controlled but cannot represent the whole picture. This is appositely illustrated by the presence of cells that fail to express both M_4 and REST/NRSF and other cells that express M_4 in the presence of REST. Nevertheless, REST/NRSF occupies a pivotal position in controlling neuronal gene expression and hence we have examined its mode of action in order to gain insight into mechanisms that may specify its action on particular genes.

The conservation of many transcriptional mechanisms between yeast and mammalian cells coupled with the small size of the yeast genome and its ease of manipulation have long made yeast an attractive model system for transcriptional studies. Against this background we reasoned that we could use yeast to explore molecular mechanisms responsible for REST/NRSF mediated repression. As a model system we have used the GAL1 promoter. In this system, the activator gal4p binds to the cUAS (Consensus Upstream Activator Sequence) upstream of the GAL1 promoter and, in the presence of galactose, activates transcription. In the absence of galactose, no activation occurs and the promoter is derepressed. In the presence of glucose, the promoter is actively repressed by the action of mig1p. This latter repression requires the SSN6/TUP1 corepressor complex. By placing RE1/NRSE elements downstream of the cUAS we have examined the ability of REST/NRSF to interact with the GAL1 promoter. This has allowed us to make several observations.

Firstly, we have shown that RE1/NRSE elements can recruit REST/NRSF to repress the gal4p activated GAL1 promoter. Secondly, we have shown that this repression is unimpaired in a SSN6 deleted isogenic strain of yeast and therefore does not require recruitment of the SSN6/TUP1 corepressor complex. Thirdly, we have shown that deletion of the sin3 corepressor abolishes the ability of REST/NRSF to repress the activated GAL1 promoter. The mammalian homologues of ysin3 have previously been shown to mediate retinoid receptor (21) and Mad/Max (22,23) repression of target genes but thus far sin3 has not been shown to play any role in repressing neuronal gene expression. In the case of the retinoid receptor and Mad/Max repression, sin3 has been shown to interact with, and require, histone deacetylase activity (21-24). Consequent deacetylation of core histones leads to local changes in chromatin architecture and nucleosomal condensation resulting in transcriptional silencing. Conservation of mechanism across yeast and mammalian systems is underwritten by the observation that the N-terminal SID (Sin3 Interaction Domain) of Mad interacts with the equivalent paired amphipathic helix domain of both yeast and mouse sin3 (25).

Furthermore we have identified two distinct repression domains and a hitherto unknown activator domain. Only one of these repression domains recruits sin3 while the other operates via an unknown mechanism. The existence of multiple domains, each recruiting different transcriptional machinery that can be directed to distinct promoters goes some way to providing a

molecular foundation to explain the diversity and selectivity of actions of REST/NRSF on the M_4 promoter. ChIPs, GST fusions and two hybrid assays are now underway to demonstrate direct interactions between members of these complexes in both yeast and mammalian cells.

E. Conclusions

Despite the gross similarity of genomic structure and expression patterns of the M_1 and M_4 genes, the cell specific expression of each of these genes appears to be controlled by quite disparate mechanisms. In the case of the M_4 promoter, a component of cell specific expression appears to arise from use of alternative promoters with differing sensitivity to the silencer, REST/NRSF. It is clear that any complete explanation of the expression patterns of these genes will entail the elucidation of multiple DNA/protein and protein/protein interactions. It is equally apparent that such elucidation will require concerted studies carried out in a range of expression vehicles from *Saccharomyces* to *Mus.*

Acknowledgement

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MUSCARINIC RECEPTOR SUBTYPES INVOLVED IN HIPPOCAMPAL CIRCUITS

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Summary

Muscarinic receptors modulate hippocampal activity in two main ways: inhibition of synaptic activity and enhancement of excitability of hippocampal cells. Due to the lack of pharmacological tools, it has not been possible to identify the individual receptor subtypes that mediate the specific physiological actions that underlie these forms of modulation. Light and electron microscopic immunocytochemistry using subtype-specific antibodies was combined with lesioning techniques to examine the pre- and postsynaptic location of m1-m4 mAChR at identified hippocampus synapses. The results revealed striking differences among the subtypes, and suggested different ways that the receptors modulate excitatory and inhibitory transmission in distinct circuits. Complementary physiological studies using m1-toxin investigated the modulatory effects of this subtype on excitatory transmission in more detail. The implications of these data for understanding the functional roles of these subtypes are discussed.

Key Words: dentate gyrus, hippocampal afferents, NMDA receptors

The hippocampal formation is a cortical structure that has been strongly implicated in the processes of memory acquisition and retention. For instance, administration of muscarinic receptor antagonists or deafferentation of cholinergic input to the hippocampus both result in a memory and attentional deficits (1-4). Muscarinic transmission is also implicated in normal aging and disease states in which cholinergic systems are disturbed and deficits in memory and

attention result (5-7). As an example, degeneration of cholinergic basal forebrain neurons which project to the cortex and hippocampus in Alzheimer's Disease (AD) has been suggested to contribute to the dementia syndrome (8).

The mechanisms by which muscarinic receptors are involved in learning and memory are not known. While muscarinic receptors have many physiological effects on cellular functions in hippocampus, it is not clear which of the receptor subtypes mediate different functions. The assignment of particular subtypes to specific physiological actions has been complicated by imperfect specificity of ligands available for the muscarinic family (9,10). To circumvent this problem we have used complementary anatomical, molecular, and physiological approaches to begin to clarify the roles of individual receptors in hippocampal functions. Subtype-specific antibodies have allowed us to identify and quantify the subtypes in different brain regions, including hippocampus (11,12). Here we review recent immunocytochemical studies at the light and electron microscopic level, which have been combined with classical lesioning approaches to define the pre- and postsynaptic localization of the receptors within identified neural circuits. The anatomical information gained was then used to guide physiological studies further testing receptor subtype functions using highly selective muscarinic toxins (13,14).

Cholinergic innervation of the hippocampus is widespread, underlying the multi-faceted effects of ACh as a modulator of hippocampal transmission. Physiological activity is initiated and propagated through the hippocampus via a series of three glutamatergic synapses (the trisynaptic pathway) (15). The major input originates from pyramidal cells in the entorhinal cortex, which collects polymodal information from other cortical areas and then projects via the perforant pathway to terminate in the molecular layer of the dentate gyrus. Information is relayed by the dentate granule cells to the CA3 region, and subsequently via Schaffer collaterals to CA1 pyramidal cells. The CA1 pyramidal cells are the major output cells for the hippocampus. Each region also has intrinsic circuits and rich connections within (associational) and between (commissural) hippocampi. Anatomical and physiological evidence suggests that ACh, acting via mAChR, can differentially modulate excitatory and inhibitory transmission at many sites along the hippocampal circuit. To clarify the synaptic organization and function of the mAChR subtypes, we focus here on several components of the circuit: 1) the cholinergic inputs via the septo-hippocampal projection; 2) the dentate gyrus, the main input receiving area of the hippocampus via the perforant pathway and also a site for many associational/commissural connections; and 3) the CA1 region, the main output area.

Cholinergic Septo-hippocampal Pathway Autoreceptors

Cholinergic input to the hippocampus derives mainly from the medial septum/diagonal band nuclei of the basal forebrain (16). Release studies have shown that muscarinic receptors play an autoregulatory role in the inhibition of ACh release in the hippocampus (17,18). However, the identity of the autoreceptor(s) in hippocampus has been controversial (17-21). Previous immunocytochemical and *in situ* hybridization studies have indicated m2 receptors are highly expressed in the cell bodies of basal forebrain cholinergic neurons, consistent with a role as autoreceptor (11). However, whether or not these receptors are transported to axon terminals in hippocampus has been unclear. Lesion studies have shown only a partial (~25%) and transient reduction in m2 receptors in hippocampus despite nearly complete deafferentation of cholinergic inputs (12,22). Immunocytochemical studies in the lesioned animals also failed to show any clear cut alterations in the distribution of m2 or other subtypes (12). To further address this issue, we performed at immunocytochemical studies to determine if m2 is present in cholinergic nerve terminals in hippocampus. Antibodies to the vesicular acetylcholine

transporter (VAChT), a protein found exclusively in cholinergic synaptic vesicles, were used to label cholinergic nerve terminals in combination with m2 receptors. By light microscopic analysis of sections processed for visualization of either marker alone, the distribution of the m2 receptor was similar to that of VAChT in dentate gyrus (Fig. 1, A-B) and other regions of the hippocampus. By electron microscopic analysis of tissue processed for co-localization of the markers, m2 was found to be presynaptic in many cholinergic axon terminals throughout the hippocampal formation (Fig. 1, C-E). These observations provide direct molecular and anatomical evidence supporting a role for m2 as an autoreceptor on cholinergic nerve terminals in hippocampus.

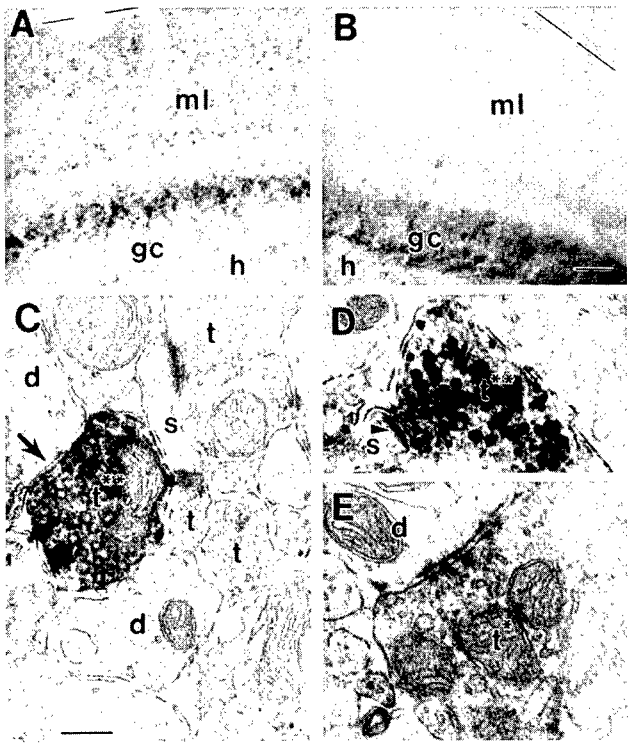

Fig. 1

Light and electron microscopic localization of VAChT **(A)** and m2 **(B)** in the dentate gyrus. Immunoreactivity for both is found in and around the granule cell layer and in the molecular layer. Double labeling for the VAChT and m2 proteins at the electron microscopic level show that they co-localize in terminal profiles in the dentate gyrus. **C:** A double labeled terminal (t**) that had m2 (gold, dark particles) and VAChT (DAB, diffuse reaction product that fills the profile) is seen here making a symmetric synapse (arrow) onto a dendrite (d) as was frequently seen in the granule cell layer. Non-immunoreactive spines (s) and dendrites (d) are also present. **D:** A double-labeled terminal (t**) that is immunoreactive for m2(DAB) and VAChT (gold) is seen here making an asymmetric synapse (arrowhead) with a dendritic spine (s), as was occasionally seen in the granule cell layer. **E:** A m2 (DAB) immunoreactive terminal (t*) in the granule cell layer that is not immunoreactive for VAChT. **ml**=molecular layer, **gc**=granule cell layer; **h**=hilus. Scale bars: A-B, 100 μm; C-D, 219 nm; E, 170nm.

mAChR in the Perforant Pathway and Associational/Commissural Projections

The perforant pathway is the main excitatory input to the hippocampus, originating in the entorhinal cortex (EC), and relaying polymodal information from other cortical areas. Perforant pathway fibers terminate in the middle and outer thirds of the molecular layer of dentate gyrus onto granule cell dendrites (23) where m1-m4 are differentially expressed in laminar patterns (12). The perforant pathway is anatomically divided into two physiologically distinct pathways. The lateral perforant path arises from pyramidal cells in the lateral entorhinal cortex and projects to the outer molecular layer, whereas the medial perforant path arises from pyramidal cells in the medial entorhinal cortex and terminates in the middle third of the entorhinal cortex (23). Physiologically these two pathways have different properties and are modulated differentially by muscarinic receptors (24-26). For instance, in guinea pig hippocampus, mAChR agonists can inhibit synaptic transmission at the medial but not the lateral perforant path (24), indicating that there may be some differential expression of mAChR subtypes in the terminals of the two subdivisions of the perforant path. Also, muscarinic receptors inhibit and/or facilitate long term potentiation (LTP) and long term depression (LTD) differentially at the two perforant pathway-granule cell synapses (26,27).

To identify presynaptic and postsynaptic mAChRs at perforant pathway synapses, we performed lesion studies in combination with light and electron microscopic immunocytochemistry for m1-m4 subtypes. The m1 receptor was found in dendrites and spines, and was unaffected by perforant pathway lesions, indicating that it is a postsynaptic receptor. On the other hand, m2, m3 and m4 were frequently found in presynaptic axon terminals. Interestingly, the composition of presynaptic subtypes differed in the medial vs. lateral division of the perforant pathway. All three subtypes were present in the medial perforant path, whereas m3 receptor was predominant in the lateral perforant path (Fig. 2) (28). The differential expression of subtypes in the two subdivisions of the perforant pathway is consistent with the distinct physiological responses of these pathways to cholinergic modulation. Functionally, these findings also suggest that all three subtypes are heteroreceptors that modulate glutamate release in the perforant pathway.

The commissural and associational pathways play an important role in integrating information along the septotemporal axis of the hippocampal formation, as well as regulating granule cell function between hippocampi (29). Both pathways extend the entire septotemporal extent of the hippocampal formation, arising from mossy cells, and CA3c pyramidal cells (30). The associational pathway also contains some gamma-aminobutyric acid (GABA)ergic neurons in the hilus (31). Both pathways specifically terminate on granule cell bodies and proximal dendrites in the inner third of the molecular layer, as well as on GABAergic hilar interneurons (32,33). Some commissural and associational pathway neurons have been identified as excitatory, utilizing either aspartate or glutamate as their neurotransmitter (34). Using lesion studies combined with light microscopic immunocytochemistry, we have identified that m2 is presynaptic on the associational pathway, whereas m4 receptors are presynaptic on both the commissural and associational pathways (Fig. 2). Hence, our results suggest that m2 may be presynaptic on the GABAergic neurons of the associational pathway, with m4 presynaptic on the excitatory amino acid containing neurons of both pathways (35). Behaviorally, the commissural and associational pathways play an important role in memory. Kainic acid lesions of hilar cells similar to the ones used in our study result in deficits of both working and spatial memory (36,37). The presence of pre-synaptic mAChRs that regulate the release of glutamate, aspartate, and GABA, may be important in integrating basal forebrain activity into memory formation and retention in the hippocampus. Therefore, presynaptic mAChRs on the commissural and associational pathways could play an important regulatory role in intrahippocampal transmission and ultimately in the formation and retention of memory.

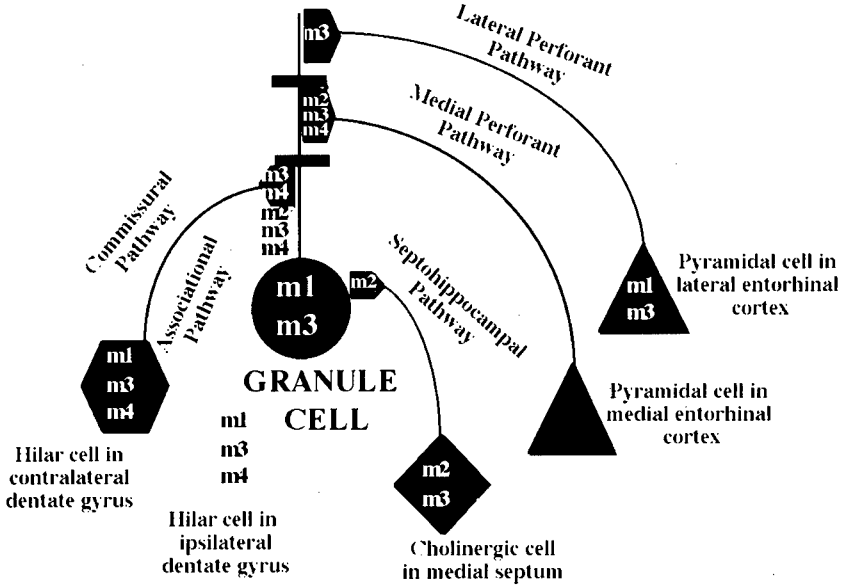

Fig. 2

Summary of the expression patterns of the m1-m4 proteins in the cell bodies and terminals of the three major inputs into the hippocampal formation through the dentate gyrus

Muscarinic modulation of the hippocampal output: excitation of CA1 pyramidal cells

The anatomical studies described above suggest that the locations of pre- and postsynaptic mAChR are well suited to modulate glutamatergic excitatory transmission. Interestingly, previous studies in cortex and striatum have revealed postsynaptic localization of m1 receptors at synapses containing excitatory amino acids (38,39). CA1 pyramidal cells are the major output cells of the hippocampus, and are modulated by mAChRs in a very different way than the perforant path input discussed above. ACh, via the activation of mAChRs, increases the excitability of pyramidal cells, in essence priming them to fire action potentials more easily in response to glutamate.

Physiological studies have demonstrated that a prominent mechanism for muscarinic receptor-mediated increases in pyramidal cell excitability is via the enhancement of the activity of the glutamatergic NMDA channel (40,41,42). In CA1 pyramidal cells, the NMDA current is increased in the presence of carbachol, a non-specific mAChR agonist. These modulatory events most likely occur in the somatodendritic regions of neurons. It is therefore reasonable to hypothesize that receptor subtypes that are expressed in these cellular compartments would be

candidates for mediators of these actions. Anatomical studies at the light and electron microscopic levels have shown that m1, and to a lesser extent, m3 receptors are localized postsynaptically in pyramidal and granule cell somata and dendrites (12,28,35). Given the physiological evidence suggesting that postsynaptic muscarinic receptors modulate NMDA receptors, we performed immuno-electron microscopic studies to determine if the m1 receptor and the NMDA receptor are co-localized in rat hippocampus (Marino et al., submitted). As shown in Fig. 3, immunoreactivity for m1 and the NR1 subunit of the NMDA receptor are co-localized in CA1 pyramidal neurons, indicating an appropriate spatial relationship for m1 to modulate the activity of the NMDA receptor.

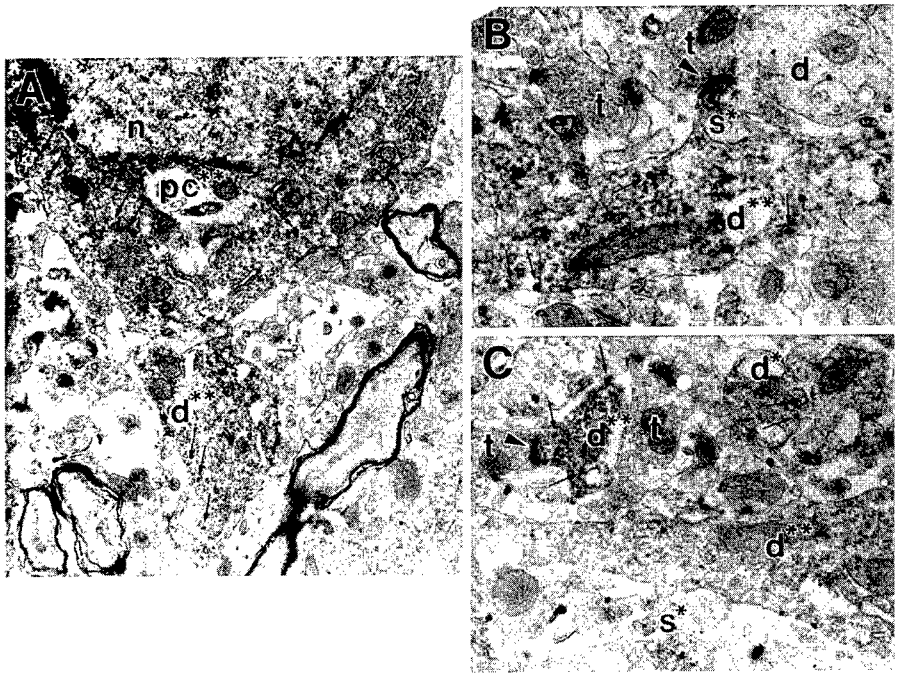

Fig. 3

The m1 receptor co-localizes with the NMDA NR1 subunit. Double labeling electron microscopic immunocytochemistry was used to identify the m1 receptor (gold) and the NR1 subunit of the NMDA receptor (DAB). **A:** A pyramidal cell soma **(pc**)** and a large dendrite **(d**)** that contain immunoreacitivty for m1 and NR1. **B-C:** Several large and small caliber dendrites are shown. Double labeled dendrites **(d**)** and spines **(s**)** can be seen, as well as single labeled spines **(s*)** that are receiving asymmetric (excitatory) synapse **(arrowhead)** from unlabeled terminals **(t)**. Scale bars: A, 1.2 mm; B, 465 nm; C, 440nm.

Physiological studies were pursued to further test the hypothesis that the m1 receptor is the molecular subtype that mediates muscarinic enhancement of NMDA currents in CA1 pyramidal neurons in hippocampal slices (Marino et al., submitted). NMDA produces inward currents in these neurons (Fig 4 A). Carbachol produced a substantial potentiation of the NMDA current. The potentiation was blocked by 1 μM atropine, indicating the effect is mediated by mAChR. The carbachol induced potentiation of NMDA currents was also blocked by the highly specific m1-toxin (13,14) (Fig 4 B, C). The effect was irreversible, indicating that it was not due to interactions with the m4 receptor. Blockade of potentiation by the m1-toxin was also not due to a non-specific toxic effect since the metabotropic glutamate receptor agonist 1S,3R-ACPD was still able to potentiate the NMDA current. Thus, the m1 mAChR subtype mediates ACh's ability to enhance NMDA currents.

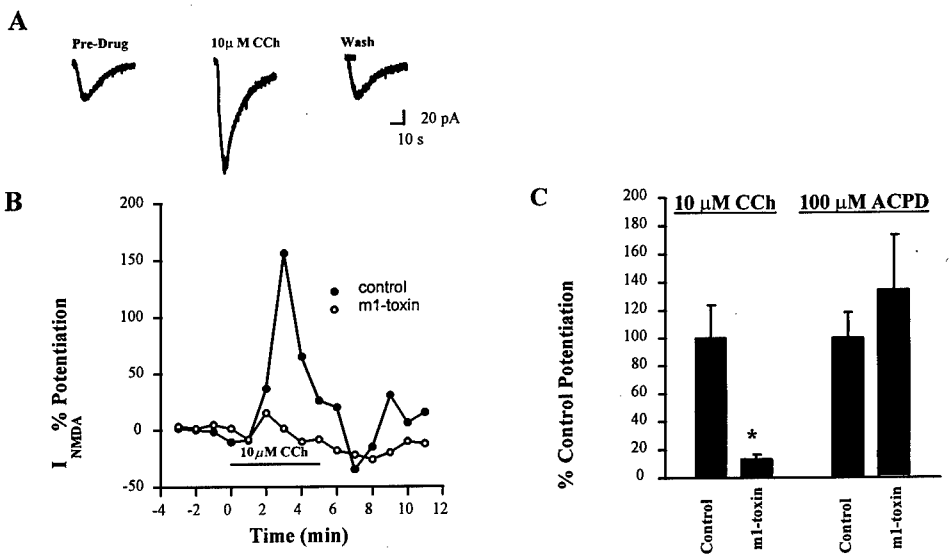

Fig. 4

Activation of the m1 mAChR potentiates NMDA-receptor currents in CA1 pyramidal cells. **A)** Bath application of 10μM CCh induces a marked potentiation of currents evoked by NMDA application. Single traces obtained before CCh application (pre-drug), at the peak of CCh-induced potentiation (10μM CCh), and after 5 min. washout. **B)** Time course of CCh-induced potentiation of CCh-induced potentiation of NMDAR current in a representative cell pre-treated with m1-toxin (open circles) and a control cell that was treated in the same fashion, but without m1-toxin (filled circles) demonstrating that the m1-toxin specifically inhibits the CCh-induced potentiation of NMDA-receptor currents. **C)** Mean (±SEM) data demonstrating that m1-toxin specifically blocks the potentiation effect of CCh, but has no effect on the response to 1S, 3R ACPD. (*$p<0.05$; n=4-5 for each condition)

Conclusion

Cholinergic modulation of hippocampus is complex, with ACh having differential actions mediated by diverse pre- and postsynaptic receptor subtypes. Anatomical studies using subtype-specific antibodies have provided a powerful approach for identifying the mAChR subtypes at defined hippocampal synapses, and for guiding physiological studies. The remarkably complementary distributions of the subtypes at presynaptic (m2, m4) vs. postsynaptic (m1, m3) sites and in distinct micro-circuits within hippocampus suggests that control of gene expression as well as intracellular trafficking of the receptors underlies the manifold modulatory effects of ACh on excitatory and inhibitory neurotransmission. The m1-mediated potentiation of NMDA receptor function has particular significance for therapeutic strategies for nervous system disorders involving hippocampal and/or glutamatergic dysfunction, including Alzheimer's disease, stroke, epilepsy, and psychoses. That is, the ability of m1 agonists to enhance NMDA receptor function may be therapeutically advantageous in disorders with reduced glutamatergic transmission, including Alzheimer's disease and schizophrenia. In contrast, such treatment might augment excitotoxicity in hypoxic-ischemic conditions or enhance excitability in epilepsy. In these latter conditions the use of m1-selective antagonists might prove effective for neuroprotection or as novel anti-epileptic agents. Further understanding of the molecular mechanisms of muscarinic functions in hippocampus will likely provide new insights into therapeutic strategies for a variety of neurological and psychiatric disorders.

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USING SINGLE PHOTON EMISSION TOMOGRAPHY (SPECT) AND POSITRON EMISSION TOMOGRAPHY (PET) TO TRACE THE DISTRIBUTION OF MUSCARINIC ACETYLCHOLINE RECEPTOR (MACHR) BINDING RADIOLIGANDS

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Summary

Two [^{18}F] labeled ligands for the mAChR were prepared and evaluated in rodents and nonhuman primates. The properties of both compounds, one an agonist and the other an antagonist, were consistent with M_2 subtype specificity.

Key Words: mAChR binding radioligands, SPECT, PET

The use of the tracer principle, first enunciated by Georg de Hevesy, has led to the widespread study of biochemical phenomena in vitro and in vivo. Those studies have been expanded more recently to include the use of radiotracers in vivo, based on the development of radiolabeled biochemicals and the appropriate imaging devices to detect the radioactivity by external imaging. The common radionuclides for SPECT are ^{99m}Tc (gamma ray at 140 keV) and ^{123}I (gamma ray at 159 keV). For PET, the radionuclides emit a positron that annihilates to give two 511 keV gamma rays at approximately 180 degrees. The positron emitting radionuclides (with their half lives) used most frequently are: ^{15}O (2.07 min.), ^{11}C (20.4 min.), and ^{18}F(109.7 min.). The specific activities (Ci/mmol) of all these radionuclides are high because they are made by nuclear transformation, that is one element is converted into another such that, except for trace contaminants, they are carrier free. The actual specific activities for the most used PET radionuclides, ^{18}F and ^{11}C, are of the order of 1000-5000 Ci /mmol at the end of the cyclotron bombardment. In vivo studies are often proposed based on in vitro assays and ex vivo assays using rodent models or autopsy data from humans. Once the biochemical pathway have been validated in humans, this approach leads to a unique procedure for monitoring biochemical changes as a function of early disease in humans. In addition, SPECT and PET have been used to assay the pharmacodynamics of potential pharmaceuticals in either non human primates or humans.

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I. The primary use of PET and SPECT radiopharmaceuticals is the study of biochemistry in the normal and diseased living subject.

The development of SPECT or PET radiotracers is a complicated procedure with additional demands beyond that required for in vitro radioligands. There are a number of issues that must be addressed before the radioligand can be used to measure a change in biochemistry as a function of disease[1]. The approaches used by various investigators to validate the radioligand include:

A. Use of the combination of the binding affinity and the binding site concentration to choose potential site-specific radiopharmaceuticals.

B. If commercially available, the use of the ^{3}H labeled parent compound to determine distribution in vivo.

C. The preparation of one or more non-radioactive derivatives.

D. The determination of the in vitro binding affinity of the parent compound and derivatives.

E. The determination of stability of the non-radioactive derivative in plasma and hepatocytes.

F. The evaluation of various physical parameters of the non-radioactive derivative (e.g., log P, protein binding).

G. In vivo displacement of the ^{3}H parent compound with the nonradioactive derivative.

H. Preparation of the radioactive derivative including active and inactive stereomers.

I. Determination of metabolites in plasma and the target organ.

J. Correlation of the distribution of the radioactive derivative in animals with binding site distribution.

K.Use of preinjection, coinjection, or postinjection to test the saturability of the binding site and the kinetics of the radioactive derivative.

L. Development of a mathematical model to interpret radioactivity measurements in terms of receptor concentration.

M. After the radioligand is validated using the above criteria, determine whether the concentration of radioactivity in the target organ is sensitive to changes in the specific binding site. If this is successful, then confirmation of the animal distribution of the radioactive derivative in humans is warranted.

Our particular interest is in a ligand for the muscarinic acetylcholine receptor (mAChR). As early as 1973, Farrow and O'Brien published on the use of [^{3}H] labeled atropine to define mAChR(2). With the development of higher affinity and higher specific activity compounds such as ^{3}H-quinuclidinyl benzilate (QNB), receptor distribution was mapped using isolated tissue(3). The first ligand to map mAChR in a human was the radioiodinated form of QNB, 3-R-quinuclidinyl 4-S-iodobenzilate (RS IQNB)(4). The use of receptor binding radiotracers differs in one important way from those used to determine receptor concentration in vitro. The biodistribution of the radiotracer in vivo must be controlled by the receptor concentration rather than by either blood flow or transport properties. There have been a number of clinical studies using RS IQNB that indicate that the ligand is responsive to changes in receptor concentration (5,6). The most comprehensive analysis of the kinetics was carried out by Sawada et al. in rats (7). This analysis showed that the uptake in cerebrum is essentially irreversible during the first 360 min. after intravenous administration and that the rate of RS

IQNB tissue uptake depends on transport across the blood brain barrier as well as the rate of binding to the receptor. However, the later data (~24 hours) is sensitive to receptor concentration.

We recently produced a series of 4-fluoroalkyl analogues of 3-(R)-quinuclidinyl (R,S) benzilate and found that the (R)-3-quinuclidinyl (S)-4-fluoromethylbenzilate (RS-FMeQNB) had the highest M_2 selectivity (sevenfold M_2/M_1) in vitro using tissue sample assays(8). The diastereomer, (R)-3-quinuclidinyl (R)-4-fluoromethylbenzilate, had an eightfold M_1:M_2 selectivity. The proof of M_2 receptor binding in vivo was complicated by the homogenous gray matter distribution of the M_2 receptor population and the lack of other M_2 ligands that cross the blood brain barrier. Coinjection of 50 nmol RS-FMeQNB per rat decreased the concentration of RS-[^{18}F]FMeQNB at 60 min. after injection by 36-54%. If the 50 nmol of RS-FMeQNB was injected at 60 min. after the injection of RS-[^{18}F]FMeQNB, the concentration of radioactivity was decreased by only 30-50% when the animals were sacrificed 60 min. later (Table 1)(9).

TABLE 1.

Percentage Reduction of Uptake upon Coinjection or Displacement (60 min. post injection) of RS-[^{18}F]FMeQNB with 50 nmol of RS-FMeQNB in Rats.

Tissue	Coinjection (%)	Displacement (%)
Heart	92*	80*
Cortex	34	16
Hippocampus	35*	23
Caudate	36*	15
Thalamus	44*	24
Pons	54*	33*
Medulla	50*	43*
Cerebellum	71*	57*

* $p<0.01$ when %ID/g for tissue is compared to %ID/g for the same tissue after injection of RS-[^{18}F]FMeQNB alone.

The distribution of RS-[^{18}F]FMeQNB at 60 min. in rat brain was uniform, which is consistent with the known M_2 distribution. Using the data compiled by McRee et al.(10), the proportion of M_2 in the rat corpus striatum is 13%, in the hippocampus 18%, in the cortex 22%, in the thalamus 50% and in the pons/medulla 82%. For both coinjection and displacment, the high M_2 percentage tissues showed the highest decreases.

RR-[^{18}F]FMeQNB shows higher uptake in cortex, hippocampus, and caudate which are tissues with a higher percentage of M_1 binding sites whereas the uptakes in the heart, the pons, and the medulla are all lower. For the RR-[^{18}F]FMeQNB compound in the coinjection paradigm, the tissues with the lowest M_2 percentage showed a statistical decrease, but numerically there was not a major difference in percent reduction (Table 2).

TABLE 2.

Percentage Reduction of Uptake upon Coinjection or Displacement of RR-[^{18}F]FMeQNB with 50 nmol of RS-FMeQNB or RR-FMeQNB in Rats.

Tissue	Coinjection: with RS-FMeQNB(%)	with RR-FMeQNB (%)
Heart	28*	60*
Cortex	37*	21
Hippocampus	45*	25
Caudate	45*	20
Thalamus	43*	41*
Pons	47	41*
Medulla	46	35
Cerebellum	26	12

* $p<0.01$ when %ID/g for tissue is compared to %ID/g for the same tissue after injection of RS-[^{18}F]FMeQNB alone.

The heart and the cerebellum, both containing predominantly M_2 receptor subtypes, showed the greatest difference in percent reduction in that RS-[^{18}F]FMeQNB in the heart and cerebellum was reduced by 92% and 71% respectively by 50 nmol of RS-FMeQNB, whereas RR-[^{18}F]FMeQNB in the heart and cerebellum was reduced by only 60% and 12% respectively by 50 nmol of RS-FMeQNB. This indicates that the two diastereomers have different subtype selectivity in vivo. The differences are not greater because the 50 nmol co-injection is relatively large and the subtype selectivity is only of the order of 7-8 fold. The muscarinic agonist, (3-(propylthio)-1,2,5-thiadiazol-4-yl)-tetrahydro-1-methyl-pyridine (PTZTP), did not compete with RS-[^{18}F]FMeQNB for M_2 binding sites in the rat brain. RS-[^{18}F]FMeQNB uptake in the monkey brain studied with PET was rapid and high in the cortex and thalamus and lower in the cerebellum. There was still uptake in the myocardium at 30-40 min. after injection. Injecting 198 nmol (67 mg)/Kgof R-QNB at 60 min. after injection accelerated the net efflux from the cortex and thalamus. Using the uptake values in the cortex and assuming that the co-injection will not affect this value, we calculate that the original concentration in the cortex after the no carrier added injection of RS-[^{18}F]FMeQNB was approximately 0.3 pmol/cc whereas after the co-injection, the concentration of RS-[^{18}F]FMeQNB and RS-FMeQNB was about 1000 times higher or 0.3 nmol/cc. If the concentration of M_2 receptors in monkey cortex is between 10 and 20 nM, then the concentration of RS-FMeQNB would be in excess of the receptor concentration. These ligands do display differential subtype selectivity and may prove useful for the in vivo imaging of muscarinic subtypes. The evidence for saturable binding and M_2 subtype selectivity for RS-[^{18}F]FMeQNB is:

- In the NovaScreen assay using brain and heart tissue, RS-FMeQNB showed a Ki of 0.9 nM for M_1 and 0.13 nM for M_2 whereas RR-[^{18}F]FMeQNB showed a Ki of 0.1 nM for M_1 and 0.8 nM for M_2. Neither bound to M_3. RS-FMeQNB and RR-FMeQNB did not inhibit binding at other biogenic amine receptors.
- The biodistribution in rats showed uniform uptake in brain structures and moderate clearing over two hours.
- RS-[^{18}F]FMeQNB was inhibited by co-injection of RS-FMeQNB at 5, 50, and 500 nmol/rat in a dose dependent manner.

- Imaging with RS-[^{18}F]FMeQNB in monkeys showed rapid and relatively uniform uptake in all brain regions with slow clearance.
- Injection of 198 nmol/Kg of RS-FMeQNB at 60 min. post RS-[^{18}F]FMeQNB showed a clear break in the brain radioactivity curve with displacement in the cerebellum and thalamus with less displacement in the cortex.
- Using an autoradiographic approach, 75 nmol of RS-FMeQNB was shown to compete with radioiodinated RS-IQNB. Data from autoradiographs taken two hours after injection of radioiodinated RS-IQNB with and without RS-FMeQNB showed a pattern of inhibition in those regions enriched in the M_2 subtype such as thalamic nuclei, pons, and the inferior superior colliculi.

However, the low net efflux of RS-[^{18}F]FMeQNB, within the time constraints imposed by the half life of ^{18}F, may limit the ability to determine the kinetic parameters necessary to evaluate the change in receptor concentration as a function of disease. An accurate input function may permit the determination of the receptor concentration even in this situation.

We have also been working with a series of muscarinic agonists first proposed by Novo-Nordisk. One of these is in clinical trials in patients with Alzheimer's disease under the USAN name of Xenomeline. This is a M_1 selective ligand whereas we have chosen a M_2 selective ligand for use in imaging deficiencies in M_2 receptors. Sauerberg et al. have shown that one compound (3-(propylthio)-1,2,5-thiadiazol-4-yl)-tetrahydro-1-methylpyridine (P-TZTP) is M_2 selective by comparing inhibition of twitch height in rabbit vas deferens (low) and inhibition of force of contraction in guinea pig atria (50 nM)(11). In the NovaScreen assay using brain and heart tissue, P-TZTP showed a Ki of 23 nM for M_1 and 1.5 nM for M_2. Our lead compound: (3-(3-fluoropropylthio)-1,2,5-thiadiazol-4-yl)-tetrahydro-1-methyl-pyridine (FP-TZTP) showed a Ki of 7.4 nM for M_1 and 2.2 nM for M_2. Neither bound to M_3. FP-TZTP did not inhibit binding at other biogenic amine receptors(12).

TABLE 3.

Distribution of [^{18}F]FP-TZTP at 60 min. in Rats as a Function of FP-TZTP Inhibitor Concentration (%ID/g).

Tissue	no-carrier-added	5 nmol FP-TZTP	50 nmol FP-TZTP	500 nmol FP-TZTP
blood	0.126 ± 0.012	0.121 ± 0.011	0.112 ± 0.002	0.123 ± 0.008
heart	0.171 ± 0.010	0.136 ± 0.012	0.121 ± 0.004	0.123 ± 0.008
bone	0.501 ± 0.055	0.520 ± 0.034	0.519 ± 0.036	0.441 ± 0.032
lung	0.372 ± 0.036	0.290 ± 0.012	0.206 ± 0.013	0.198 ± 0.012
cortex	0.484 ± 0.154	0.315 ± 0.052	0.115 ± 0.006	0.080 ± 0.005
hippocampus	0.429 ± 0.078	0.408 ± 0.072	0.160 ± 0.022	0.106 ± 0.008
striatum	0.448 ± 0.021	0.342 ± 0.062	0.146 ± 0.012	0.092 ± 0.009
thalamus	0.413 ± 0.070	0.292 ± 0.036	0.121 ± 0.015	0.086 ± 0.017
pons	0.419 ± 0.048	0.370 ± 0.120	0.135± 0.062	0.083 ± 0.014
medulla	0.529 ± 0.075	0.469 ± 0.083	0.158 ± 0.016	0.074 ± 0.006
cerebellum	0.382 ± 0.047	0.283 ± 0.048	0.128 ± 0.016	0.088± 0.004

The uptake in rat brain at early times was similar to that obtained with RS-[^{18}F]FMeQNB, but the net efflux was faster for [^{18}F]FP-TZTP. Autoradiography using no carrier added [^{18}F]FP-TZTP confirmed the uniform distribution of radioactivity which is characteristic of the pattern of localization of the M_2 antibody. At one hour after injection, co-injection of P-TZTP at 5, 50 and 500 nmol per rat inhibited uptake is a dose dependent manner (Table 3). In the brain regions, the statistical difference between each dose level was $p < 0.01$ except for the 5 nmol value for the medulla which was $p < 0.06$. The brain distribution of [^{18}F]FP-TZTP was unaffected by coinjection of 5, 50, or 500 nmol of RR-IQNB.

Likewise, the brain distribution of [^{18}F]FP-TZTP was unaffected by coinjection of 500 nmol of the M_2 selective antagonist RS-FMeQNB except in the cortex and hippocampus where the difference was statistically significant at the $p < 0.03$. We also conducted competition studies using the muscarinic ligand L-687,306 (-3(3-cyclopropyl-1,2,4-oxadiazol-5-yl)-1-azabicyclo[2.2.1]heptane) and found that at a dose of 500 nmol per rat, the concentration of [^{18}F]FP-TZTP was decreased in all brain regions. Binding in the heart was low by 15 min. so earlier time points were studied. At 5 minutes, we observed 55% inhibition of uptake in the heart with co-injection of P-TZTP.

Biodistribution studies in monkeys showed similar results. [^{18}F]FP-TZTP was rapidly taken up with uniform clearance throughout the brain except for the cerebellum which cleared more slowly. Displacement of [^{18}F]FP-TZTP with 80 nmol/Kg of FP-TZTP at 45 min. post injection caused a distinct change in the net efflux with percentage decreases of 20, 36, and 41 in cerebellum, cortex, and thalamus, respectively(13).

A major concern in studies with [^{18}F]FP-TZTP is metabolism. In order to accurately determine the binding potential, defined as the product of the affinity constant and the free receptor concentration, the time course of the parent compound, [^{18}F]FP-TZTP, in the blood must be determined. This is carried out by rapid blood sampling, mixing with acetonitrile, and analysis of the acetonitrile/water using either TLC or HPLC. The parent compound degrades quickly in vivo, representing 62, 23, and 11% of extracted monkey plasma radioactivity at 15, 30, and 90 minutes. The amount of parent in rat plasma decreased to 5% by 15 min. post injection. Although there was a metabolite nearly as lipophilic as the parent compound on TLC, the parent compound in the rat brain represents greater than 95% of the extracted radioactivity through 30 min. and greater than 90% at 45 and 60 min. The evidence from all experiments indicate that [^{18}F]FP-TZTP is a M_2 selective radioligand with reversible pharmacokinetics and predominately parent compound in the target organ. Human studies are now in progress.

II. A second use of PET and SPECT radiopharmaceuticals is in the evaluation of new drugs. There are a number of ways that such radiotracers have been used:

A. The Effect of Pharmaceuticals on Organ Perfusion

There are many well established radiopharmaceuticals that are capable of measuring flow in a particular organ using external detection. The flow can be quantified, i.e. put in terms of vol/time, in special cases, but in general relative measurements are made. These measurements can be made with substances that remain in the vascular compartment or with substances that diffuse from the vascular compartment. The advantages and disadvantages of various approaches are discussed by Lassen and Perl (14).

B. The Effect of Pharmaceuticals on Cognitive and Somatosensory Function. A growing body of literature attests to the sensitivity of PET and SPECT for measuring the neurophysiological concomitants of a subject's mental state and behavior, sensory input, motor outputs, and cognitive activity. As a consequence of this unique capability, these tools are currently the most powerful in the armamentarium for investigating the normal human cerebral functional landscape, the physiological responses of the human brain to the challenges of daily activities, and its functional characteristics under pathological conditions (15,16).

C. The Analysis of Relative Potency of Putative Pharmaceuticals on a Molecular Basis. In the past it has been the practice to radiolabel each new pharmaceutical to test its efficacy in vivo as a diagnostic imaging agent. However, in the context of developing a procedure to analyze the relative potency of putative pharmaceuticals, only one radiolabeled ligand is needed. The relative potency of the others can be established once the radiolabeled compound is defined by using relative competition for a limited number of binding sites. These studies are first carried out in nonhuman primates and then in humans.

D. The Analysis of Target Site Occupancy As a Function of Time. To date, most new drugs have been developed on the basis of animal studies using ^{14}C and ^{3}H labeled forms of the drug. In addition a surrogate marker of the biochemical action is measured in animals. The development of SPECT and PET techniques to measure the pharmacokinetics in vivo in humans by non-invasive imaging will lead to better understanding of the drug of choice.

[^{18}F]FP-TZTP can be used to monitor changes in neurotransmitter in vivo using PET imaging and thus supply information on the relative potency of putative pharmaceuticals involved in the concentration of extraneuronal neurotransmitter. For example, we have shown that [^{18}F]FP-TZTP can monitor the increases in acetylcholine as a function of infused physostigmine(17). Physostigmine was administered by i.v. infusion of 100 to 200 mg/Kg/h beginning at 30 min. before [^{18}F]FP-TZTP injection. Likewise, the concentration of [^{18}F]FP-TZTP in the brain regions can be blocked by preinjecting relatively large amounts of nonradioactive receptor binding ligand. The former experimental paradigm could be used to test various phosphodiesterase inhibitor drug candidates whereas the latter paradigm could be used to test the relative potency and selectivity of various M_2 specific ligands. The volume of distribution (V) is the equilibrium tissue to plasma concentration ratio was measured for this reversible ligand. V is a function of the free receptor concentration and the receptor dissociation equilibrium constant. The specific volume of distribution (V_s) can be obtained by subtracting the volume of distribution obtained with saturating ligand (a measure of nonspecific binding) from the volume of distribution. The V_s for the no-carrier added [^{18}F]FP-TZTP, for preblocking of [^{18}F]FP-TZTP with 200-400 nmol/Kg of FP-TZTP, and for the infusion of physostigmine is given in Table 4.

All regions showed significant decreases ($p < 0.05$) in V as a function of the pharmacologic intervention with physostigmine except for the cerebellum, basal ganglia, thalamus, and temporal cortex. [^{18}F]FP-TZTP is taken up rapidly with about 0.045 %ID/cc in the brain at ~20 min. Therefore, for the 200-400 nmol/Kg and assuming 8 Kg monkeys, the concentration of FP-TZTP will be ~1 mM. The highestconcentration of mACh M_2 receptor in the monkey brain is 24 nM and for mACh M_1 51 nM. Physostigmine is known to increase the concentration of acetylcholine by 1000% as measured by microdialysis in rats. In general, [^{18}F]FP-TZTP binding is sensitive to competition either by receptor binding agonists or by endogenous acetylcholine and may serve as a surrogate marker for the screening of potential pharmaceuticals. Both SPECT and PET are powerful tools for the measurement in vivo of easily saturated sites by external imaging

TABLE 4.
The Volume of Distribution (mL/mL) for [^{18}F]FP-TZTP under control and two blocking conditions.

Region	No-carrier-added	preblock w. FP-TZTP	physostigmine
Cerebellum	15.7 ± 3.8	7.6 ± 0.5	13.6 ± 4.1
Basal Ganglia	25.6 ± 5.8	9.2 ± 0.7	23.7 ± 7.0
Thalamus	23.2 ± 6.1	9.2 ± 0.7	18.9 ± 5.5
Temporal Cortex	24.4 ± 5.3	8.8 ± 0.9	20.5 ± 6.0
Occipital Cortex	21.7 ± 6.0	9.0 ± 1.2	16.9 ± 4.8
Parietal Cortex	25.5 ± 6.9	8.9 ± 1.1	18.2 ± 4.5
Frontal Cortex	26.5 ± 7.5	8.2 ± 0.8	20.5 ± 5.8
Average Cortex	24.5 ± 6.0	8.7 ± 0.9	18.9 ± 5.1

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ALLOSTERIC EFFECTS OF FOUR STEREOISOMERS OF A FUSED INDOLE RING SYSTEM WITH ^{3}H-N-METHYLSCOPOLAMINE AND ACETYLCHOLINE AT M_1-M_4 MUSCARINIC RECEPTORS

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Summary

We previously demonstrated that brucine and some analogues allosterically enhance the affinity of ACh at muscarinic receptor subtypes M_1, M_3 or M_4. Here we describe allosteric effects at human M_1 - M_4 receptors of four stereoisomers of a pentacyclic structure containing features of the ring structure of brucine. All compounds inhibited ^{3}H-NMS dissociation almost completely at all subtypes with slopes of 1, with similar affinity values at the ^{3}H-NMS-occupied receptor to those estimated from equilibrium assays, consistent with the ternary complex allosteric model. Compound **1a** showed positive cooperativity with ^{3}H-NMS and small negative or neutral cooperativity with ACh at all subtypes. Its stereoisomer, **1b**, showed strong negative cooperativity with both ^{3}H-NMS and ACh across the subtypes. Compound **2a** was positive with ^{3}H-NMS at M_2 and M_4 receptors, neutral at M_3 and negative at M_1 receptors; it was negatively cooperative with ACh at all subtypes. Its stereoisomer, **2b**, was neutral with ^{3}H-NMS at M_1 receptors and positive at the other subtypes; **2b** was negatively cooperative with ACh at M_1, M_3 and M_4 receptors but showed 3-fold positive cooperativity with ACh at M_2 receptors. This latter result was confirmed with further ^{3}H-NMS and ^{3}H-ACh radioligand binding assays and with functional assays of ACh-stimulated ^{35}S-GTPγS binding. These results provide the first well characterised instance of a positive enhancer of ACh at M_2 receptors, and illustrate the difficulty of predicting such an effect.

Key Words: **allosterism, ACh, muscarinic receptors, indoles, stereoisomers**

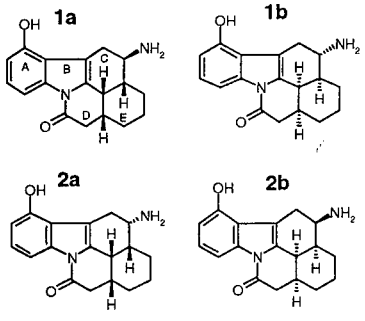

Fig. 1

In addition to the 'primary' ligand binding site for ACh and competitive antagonists, muscarinic receptors also contain one or more 'allosteric' sites to which ligands can bind simultaneously with ligands binding to the primary site. According to the ternary complex allosteric model (1,2) binding of an allosteric ligand can modify the affinity of a primary ligand, and this cooperative effect depends crucially on the particular ligands, as well as on the receptor subtype. We have reported previously that brucine (10,11-dimethoxy strychnine) binds allosterically to the M_1 receptor and increases the affinity of ACh for the M_1 receptor by about 2-fold (3,4), and this finding has been independently confirmed (5). We also found that N-substituted brucine analogues showed different patterns of cooperativity with the directly acting ligands ACh and ^{3}H-NMS across the

muscarinic receptor subtypes. Here we describe the allosteric effects of four stereoisomers (Fig.1) which contains features of the ring structure of brucine.

Methods

The methods have been described in detail (2,4). Briefly, membranes were prepared from CHO cells expressing individual human muscarinic M_1-M_4 receptors. Data from equilibrium binding assays with ^{3}H-NMS and ACh were fitted to the allosteric model, with a kinetic adjustment if necessary. Data from assays measuring inhibition of ^{3}H-NMS dissociation were fitted to a hyperbolic function and, according to the allosteric model (2) the IC_{50} of an agent in these assays corresponds to the Kd of the agent for the ^{3}H-NMS-occupied receptor.

1a and **1b** (compounds (+)10 and (-)10 respectively in (6)) were synthesised as antiarrythmic agents; **2a** and **2b** were synthesised by Sankyo chemists as described (7).

Results

All compounds caused approximately complete inhibition of ^{3}H-NMS dissociation from M_1-M_4 receptors (Table I). The most potent was **2b** with log affinities of about 5.5, followed by **1a** and **2a** with log affinities of about 4.5, and **1b** was the weakest with log affinities of about 3.7. No compound showed marked receptor subtype selectivity for this effect. These directly observed affinities for the ^{3}H-NMS-occupied receptors are in excellent agreement with values estimated from equilibrium assays (calculated as pK + log α from the data summarised in Table II), indicating that the data from both types of assay are internally consistent with the ternary complex allosteric model.

TABLE I

Log Affinity at ^{3}H-NMS-occupied Receptors and Maximal Inhibition of ^{3}H-NMS Dissociation Rate.

Compound		M_1	M_2	M_3	M_4
1a	pK	4.84 ± 0.05 (2)	4.44 ± 0.06 (2)	3.99 ± 0.11 (2)	4.61 ± 0.04 (2)
	Emax	94.2 ± 0.6	92.8 ± 0.5	100.1 ± 3.0	97.5 ± 0.6
	pKest	4.82 ± 0.01 (2)	4.58 ± 0.10 (2)	4.06 ± 0.09 (2)	4.65 ± 0.03 (3)
1b	pK	4.05 ± 0.26 (2)	4.00 ± 0.14 (2)	3.91 ± 0.17 (2)	4.24 ± 0.25 (2)
	Emax	98.4 ± 1.8	92.7 ± 2.3	99.9 ± 3.1	100.5 ± 3.7
2a	pK	4.55 ± 0.03 (2)	4.89 ± 0.07 (2)	4.69 ± 0.16 (2)	4.90 ± 0.17 (2)
	Emax	97.5 ± 1.9	107.4 ± 2.7	99.1 ± 2.5	103.3 ± 2.1
	pKest	3.83 ± 0.15 (2)	5.54 ± 0.26 (2)	4.79 ± 0.03 (2)	5.17 ± 0.07 (2)
2b	pK	5.54 ± 0.02 (2)	5.42 ± 0.03 (2)	5.30 ± 0.03 (2)	5.71 ± 0.04 (2)
	Emax	93.8 ± 2.7	100.5 ± 0.4	95.0 ± 0.8	97.5 ± 0.3
	pKest	5.60 ± 0.06 (4)	5.64 ± 0.17 (3)	5.22 ± 0.08 (4)	5.59 ± 0.23 (2)

Data are mean ± s.e.m from (n) assays. Slopes were not different from 1, and were fixed at 1. pKest is the estimated log affinity at the ^{3}H-NMS-occupied receptor from equilibrium assays summarised in Table II: values could not be calculated for **1b**

The compounds showed a different pattern of affinity values at the unliganded receptors (Table II). The most potent was **1b**, which also showed a degree of receptor subtype selectivity, with log affinity values of about 7 at the M_1 receptor and 5 at the M_2 receptor. The other three compounds did not show marked receptor subtype selectivity and had log affinity values for the free receptor in the range 4.5 - 5.5, with **1a** 3-10-fold weaker than **2a** and **2b**.

The differences in affinity between free and ^{3}H-NMS-occupied receptors led to strikingly different patterns of cooperativity with ^{3}H-NMS. **1a** was positive with ^{3}H-NMS at all subtypes (Fig.2), while **1b** appeared to be a competitive antagonist at all subtypes (Fig.3). **2a** was strongly negative at M_1 receptors, positive at M_2 receptors, and weakly negative or neutral at M_3 and M_4 receptors (Fig.4). **2b** was neutral at M_1 receptors and positive at M_2-M_4 receptors (Fig.5).

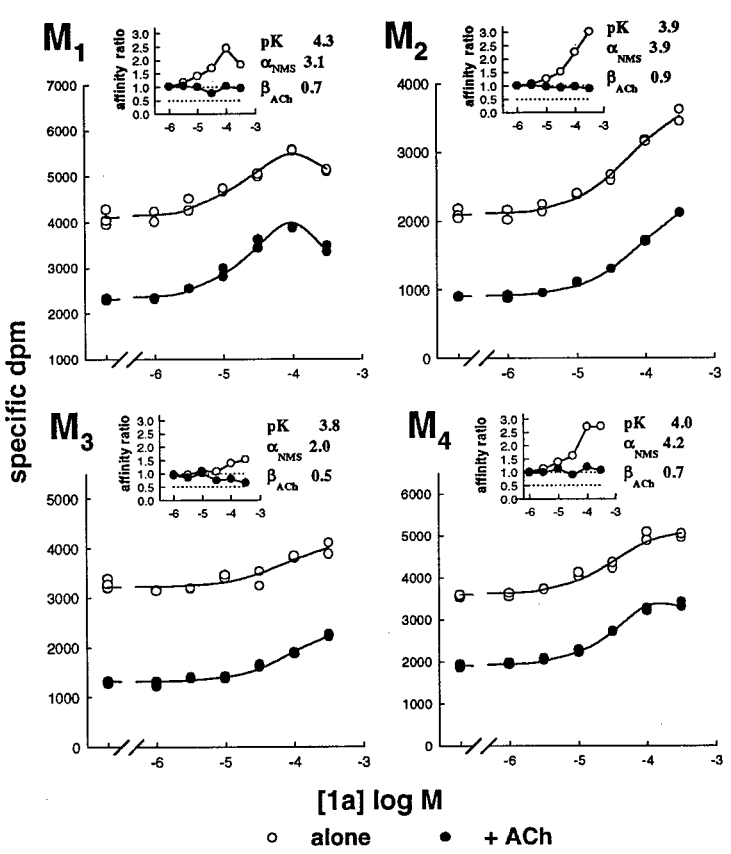

Fig. 2
Effect of **1a** on ^{3}H-NMS binding ± ACh. 0.2 mM GTP was present. *Data points* are individual replicates. *Lines* show the fit to the allosteric model, which includes a kinetic component. *Insets* show calculated affinity ratios. The *values* to the right are the parameter estimates from the fit.

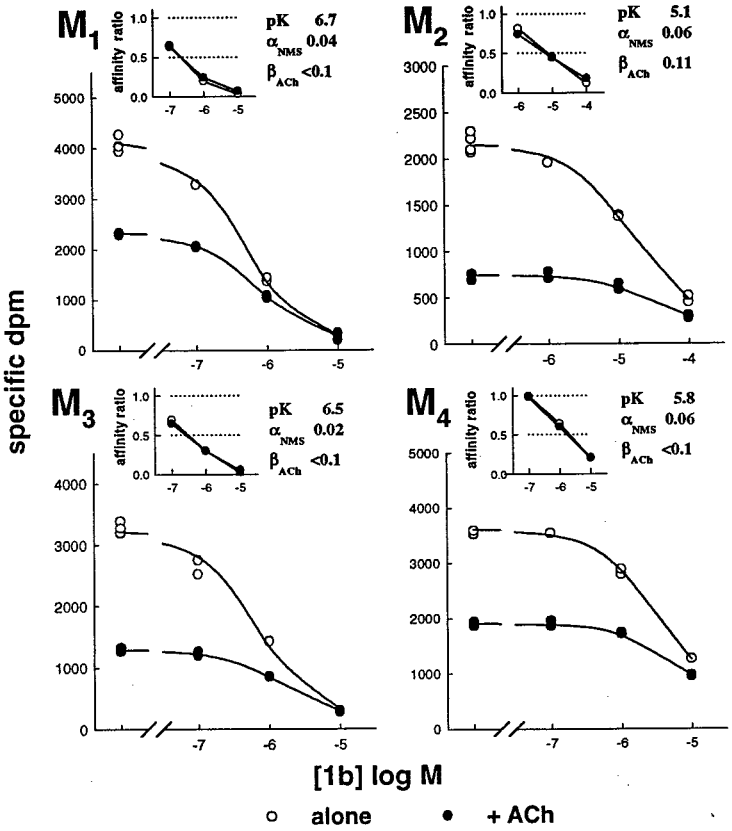

Fig. 3
Effect of **1b** on ^{3}H-NMS binding ± ACh.
See caption to Figure 2 for details.

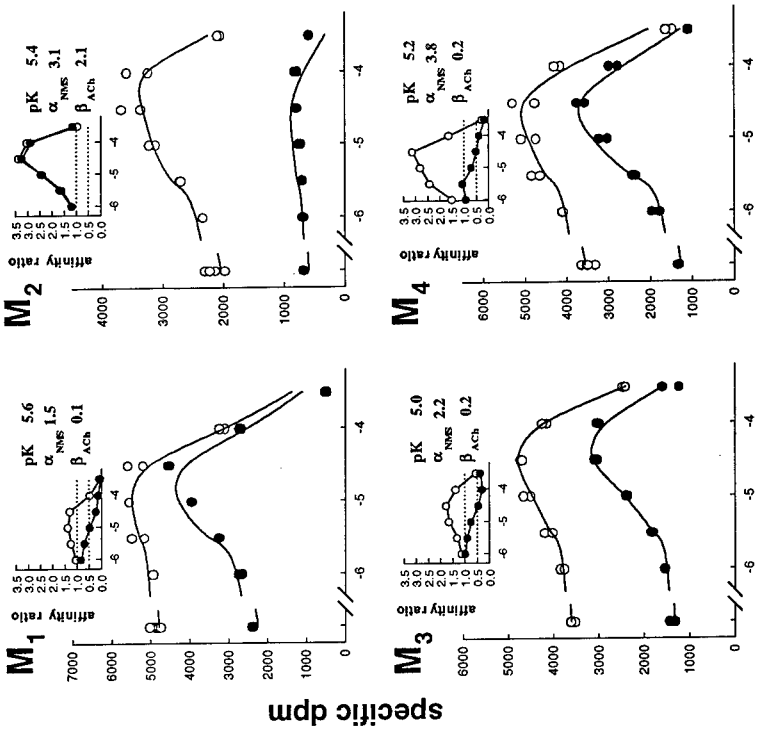

Fig. 4
Effect of **2a** on ^{3}H-NMS binding ± ACh
See caption to Figure 2 for details.

Fig. 5
Effect of **2b** on ^{3}H-NMS binding ± ACh
See caption to Figure 2 for details.

The cooperativity of the compounds with unlabelled ACh showed a different pattern. Both **1b** and **2a** were strongly inhibitory with ACh at all subtypes, **1a** was neutral at all subtypes, while **2b** was negative at M_1, M_3 and M_4 receptors, and positive at M_2 receptors (Figs.2-5).

TABLE II

Log Affinity for the Free Receptor, Cooperativity with ^{3}H-NMS (α) and with ACh (ß).

Compound		M_1	M_2	M_3	M_4
1a	pK	4.30 ± 0.02 (2)	4.06 ± 0.17 (2)	3.72 ± 0.12 (2)	4.06 ± 0.06 (3)
	α	3.3 ± 0.2	3.4 ± 0.5	2.2 ± 0.2	4.0 ± 0.1
	ß	0.69 ± 0.05	0.83 ± 0.05	0.46 (n=1)	0.84 ± 0.10
1b	pK	6.90 ± 0.10 (4)	4.93 ± 0.19 (2)	6.45 ± 0.04 (2)	5.95 ± 0.11 (2)
	α	0.007 ± 0.013	0.06 (n=1)	0.01 ± 0.07	0.04 ± 0.02
	ß	0.01 (n=1)	0.11 (n=1)	0.07 (n=1)	0.15 ± 0.11
2a	pK	5.06 ± 0.23 (2)	5.14 ± 0.28 (2)	4.89 ± 0.10 (2)	5.03 ± 0.12 (2)
	α	0.06 ± 0.01	2.54 ± 0.07	0.80 ± 0.13	1.40 ± 0.16
	ß	0 ± 0	0.12 ± 0.02	0.05 ± 0.00	0.02 ± 0.02
2b	pK	5.55 ± 0.05 (4)	5.17 ± 0.12 (4)	4.91 ± 0.08 (4)	5.09 ± 0.07 (3)
	α	1.14 ± 0.13	3.02 ± 0.04 (3)	2.03 ± 0.11	3.16 ± 0.65 (2)
	ß	0.13 ± 0.03	3.10 ± 0.85	0.18 ± 0.06	0.16 ± 0.09

Data are mean ± s.e.m from (n) assays. Slopes were not different from 1, and were fixed at 1, except for one assay with **2a** at M_2 receptors, where a value of 1.4 was estimated. Values for **2b** at M_2 receptors were from three assays with ^{3}H-NMS and one with ^{3}H-PrBCh. In a few assays parameter values could not be resolved and were therefore omitted from this summary

In an attempt to determine whether the inhibitory mechanism of **1b** at M_1 receptors was competition or negative cooperativity, inhibition curves of **1b** were constructed in the presence of various concentrations of ^{3}H-NMS (Fig.6). At low ^{3}H-NMS concentrations **1b** could completely inhibit specific binding, but as the concentration of ^{3}H-NMS increased the asymptotic level of inhibition by **1b** became > 0. This is a pattern of results predicted by the allosteric model, and the data were well fitted to the model, to yield a log affinity of 6.8 and cooperativity of 0.0010; the predicted log affinity of **1b** at the ^{3}H-NMS-occupied receptor from this result is 3.8, which is close to the value of 3.8 measured in the off-rate assay (Table I). The data were also considered as a series of ^{3}H-NMS saturation curves in the presence of increasing concentrations of **1b** (with shared Bmax), and a Schild plot was constructed from the results (Fig.6). At concentrations of **1b**

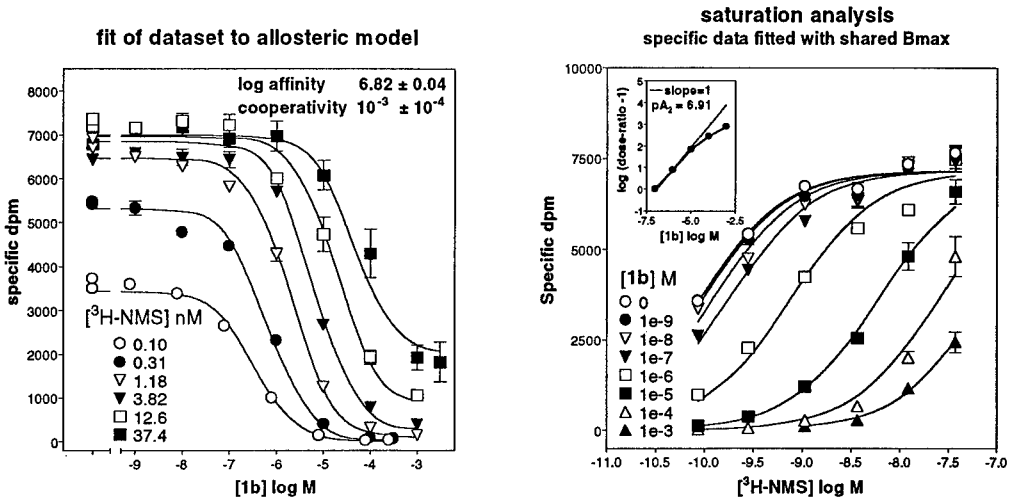

Fig. 6

Inhibition of binding of various [^{3}H-NMS] by **1b** at M_1 receptors

below 10^{-5} M the Schild plot was linear with a slope of 1, but noncompetitive results were apparent at higher concentrations. This experiment suggests that, like the other three compounds, **1b** acts allosterically, rather than, or as well as, competitively.

In earlier experiments with brucine analogues we found agents which enhanced ACh affinity at M_1, M_3 and M_4 receptors, but none at M_2 receptors, so the positive cooperativity of **2b** with ACh at M_2 receptors was of considerable interest. In indirect measures of ACh potency (in the presence of GTP) using equilibrium binding studies the positive cooperativity of **2b** with ^{3}H-NMS leads to a slowing of ^{3}H-NMS kinetics in the presence of high concentrations of **2b** (Fig.5), which complicates the interpretation of the data, so a radioligand with faster kinetics was used, ^{3}H-

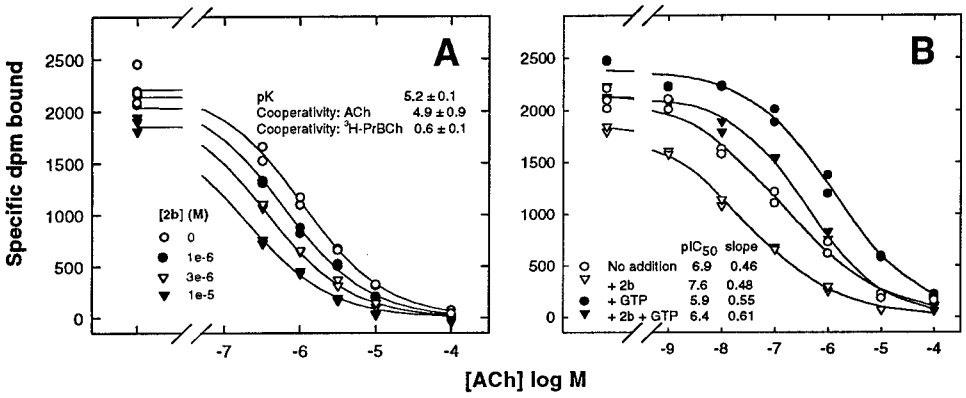

Fig. 7

A. Effect of **2b** on ACh inhibition of ^{3}H-PrBCh (8.5 nM) binding to M_2 receptors. 0.2 mM GTP was present. ^{3}H-PrBCh had a Kd of 12.7 nM in this assay B. Effect of **2b** (10 μM) on ACh inhibition of ^{3}H-PrBCh (13.5 nM) binding to M_2 receptors ± 0.2 mM GTP.

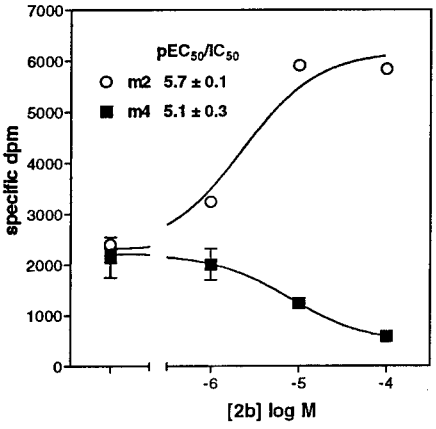

Fig. 8

Effect of **2b** on ^{3}H-ACh (2 nM) binding to M_2 and M_4 receptors

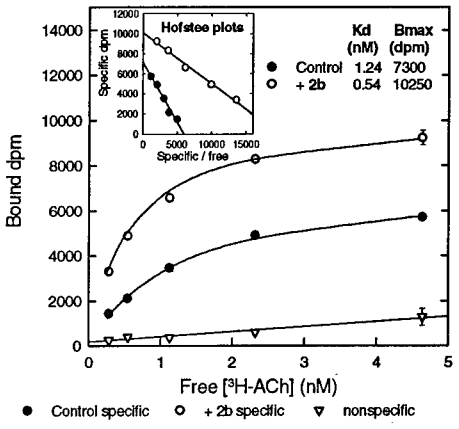

Fig. 9

Saturation binding of ^{3}H-ACh to M_2 sites in the absence and presence of 30 μM **2b**

propylbenzilylcholine (^{3}H-PrBCh). Fig.7a shows that **2b** increased the potency of ACh to inhibit ^{3}H-PrBCh binding at M_2 receptors by about 5-fold, with a small negative effect (0.6) on ^{3}H-PrBCh binding itself. **2b** had similar positive effects on ACh binding in the absence and presence of GTP (Fig.7b).

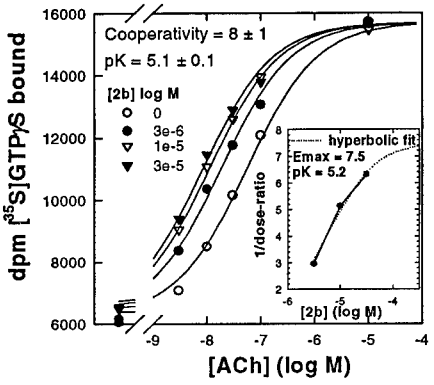

Fig. 10
Effect of **2b** on ACh stimulation of ^{35}S-GTPγS binding to M_2-containing membranes. *Lines* show the fit to the allosteric model. *Inset* shows 1/dose-ratio.

2b increased the direct binding of a single concentration of ^{3}H-ACh to M_2 receptors by up to 2.5-fold with a -log EC_{50} of 5.7 (Fig.8), which seemed to reflect increases in both the affinity and Bmax of ^{3}H-ACh (Fig.9). In contrast, **2b** inhibited ^{3}H-ACh binding to M_4 receptors (Fig.8). Figure 10 shows that concentrations of **2b** up to 30 μM increased the potency of ACh at m2 receptors for stimulating ^{35}S-GTPγS binding without affecting basal or maximal binding, while 100 μM reduced Emax and potency (not shown). The estimates of log affinity and cooperativity from this experiment are similar to the corresponding parameters measured in ^{3}H-NMS and ^{3}H-PrBCh binding assays (Table II). In another experiment 30 μM **2b** increased ACh potency at M_2 receptors by 4-fold (data not shown).

Discussion

We have previously demonstrated that brucine and its N-substituted derivatives can exhibit subtype selective positive cooperativity with ACh in binding and functional studies. (3,4). However, brucine has a very complex multicyclic ring structure. We have searched for related and, if possible, structurally and synthetically simpler molecules which exhibit higher potencies whilst retaining positive cooperativity with ACh at one or more muscarinic receptor subtypes. This would allow us to begin to explore the chemical and stereochemical features which are important for muscarinic allosteric enhancers.

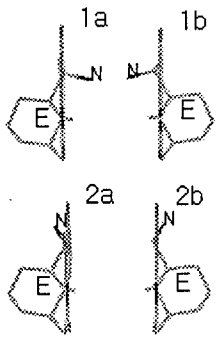

Fig. 11
3D structures viewed from the edge of ring A

We discovered that **1a**, a molecule originally synthesised as an antiarrythmic agent (6), was an allosteric agent exhibiting positive cooperativity with ^{3}H-NMS and neutral or slightly negative cooperativity with ACh at M_1-M_4 receptors. In contrast, its enantiomer **1b** was up to 500-fold more potent than **1a** at the unliganded receptor but was weaker than **1a** at the ^{3}H-NMS-liganded receptor and exhibited high negative cooperativities with both ^{3}H-NMS and ACh.

The conformations of the compounds were determined by NMR spectroscopy (H.Kuwano, B.Birdsall & N.J.M.Birdsall, unpublished results). In all the isomers, rings ABCD (Fig. 1) formed an almost planar structure with ring E perpendicular to this plane and in a chair conformation. The exocyclic amino group is axial in **1a** and **1b** and is also oriented approximately perpendicular to rings ABCD, but facing in the opposite direction to ring E. The main distinguishing feature between the enantiomers, **1a** and **1b**, is the opposite stereochemical

orientations the hydrophobic ring E and the positively charged amino group relative to the indole ring (Fig. 11). In contrast, the exocyclic amino group of **2a** and **2b** is in the equatorial position and almost coplanar with rings ABCD: the major difference between **2a** and **2b** is the direction which ring E points relative to rings ABCD. There are only small differences in the binding properties of **2a** and **2b** at the unliganded and NMS-liganded M_1-M_4 receptors and at the ACh liganded M_1, M_3 and M_4 receptors (Figs 4 and 5). However the very different cooperativities of **2a** and **2b** with ACh at the M_2 receptor imply that ring E of **2b** is making a very specific and different interaction at this subtype. The positive cooperativity of **2b** with ACh was confirmed in functional and further indirect and direct binding studies with ACh and represents the first well-characterised M_2 selective enhancer. ((-)-Eburnamonine, a compound related to the structures described here has been reported to increase ACh binding at M_2 and M_4 receptors (5) but we find it to be negatively cooperative with ACh at M_1 - M_4 receptors, n=2).

In conclusion, we have examined the allosteric properties of four optical isomers of a relatively rigid conformationally constrained structure related to that of brucine. The observed affinities and cooperativities vary with the isomer and subtype. The relatively simple structure of 2b and its micromolar potency and high selectivity for one ACh liganded muscarinic receptor subtype represents an advance in our search for potent allosteric enhancers of muscarinic receptors.

Acknowledgements

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POTENTIAL ROLE OF MUSCARINIC RECEPTORS IN SCHIZOPHRENIA

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Summary

The role of muscarinic receptors in schizophrenia was investigated using the muscarinic agonist PTAC. PTAC was highly selective for muscarinic receptors, was a partial agonist at muscarinic M_2/M_4 receptors and an antagonist at M_1, M_3 and M_5 receptors. PTAC was highly active in animal models predictive of antipsychotic behavior including inhibition of conditioned avoidance responding in rats and blockade of apomorphine-induced climbing behavior in mice. d-Amphetamine-induced Fos expression in rat nucleus accumbens was inhibited by PTAC, thus directly demonstrating the ability of PTAC to modulate DA activity. In electrophysiological studies in rats, PTAC acutely inhibited the firing of A10 DA cells and after chronic administration decreased the number of spontaneously firing DA cells in the A10 brain area. However, PTAC did not appreciably alter the firing of A9 DA cells. Thus, PTAC appears to have novel antipsychotic-like activity and these data suggest that muscarinic compounds such as PTAC may represent a new class of antipsychotic agents.

Key Words: **muscarinic receptor, muscarinic agonist, antipsychotic agent, schizophrenia, dopamine**

Schizophrenia is a severe psychosis that strikes generally in the late teens or early adulthood and occurs across all races and social classes at about 1% incidence. The major symptoms of schizophrenia have been classified as positive, negative and cognitive symptoms. Positive symptoms include distorted sensory perceptions resulting in delusions and hallucinations, whereas poverty of thought and speech, anergia, anhedonia and poor social interactions are some of the negative symptoms. Cognitive dysfunction involves decrements in attention, working memory and executive function and suggests a deficit in information processing in the cerebral cortex. The etiology of schizophrenia is unknown, but a prevailing theory for the cause of schizophrenia is the dopamine (DA) hypothesis of schizophrenia which states that schizophrenia is the result of excessive activity at DA synapses particularly in mesolimbic brain regions (1). Traditionally, pharmacotherapy of schizophrenia has consisted of treatment with DA antagonists, but they have limited efficacy as well as serious side effects including movement disorders called extrapyramidal side effects (EPS). Recently, a new class of antipsychotic agents termed atypical antipsychotics has been introduced which have marked improvement in efficacy and side effects

(2), but new pharmacologic approaches are still needed for the improved treatment of schizophrenia.

Although the neurotransmitter DA has been regarded as the key neurotransmitter involved in the pathogenesis of schizophrenia, there are a number of anatomical, pharmacological and clinical findings suggesting a role of cholinergic neurons in schizophrenia. The extensive number of cholinergic projections in human cortex suggest a potential for regulation of sensory afferents (3) and muscarinic M_1, M_2 and M_4 receptors have been found in high density in human cortex (4). The M_1 receptor, in particular, may be involved in modulation of excitatory amino acid neurotransmission in cortical and limbic areas (5). There is also considerable evidence demonstrating extensive interactions between the DA and cholinergic systems. The mRNA for muscarinic and DA receptors are colocalized in dopaminergic neurons (6,7) and dopaminergic and cholinergic receptors reciprocally modulate the release of one another (8,9). Muscarinic agonists or cholinesterase inhibitors injected near the ventral tegmental area (VTA) DA cells depolarize the DA cells (10), demonstrating cholinergic modulation of the mesolimbic DA tracts thought to be involved in schizophrenia. High doses of some muscarinic antagonists produce psychotic-like symptoms such as delusions, hallucinations and memory loss (11), suggesting a role for the cholinergic system in processing sensory information and in schizophrenia. Moreover, several clinical studies have indicated that cholinomimetics may reduce the symptoms of schizophrenia (12-15). Recently, the cholinesterase inhibitor physostigmine has been shown to reduce delusions in Alzheimer's patients (16) and the muscarinic agonist xanomeline reduced several psychotic-like symptoms in Alzheimer's patients (17). Therefore, several investigators have proposed that cholinergic agents may have a role in the treatment of schizophrenia (15, 17-19).

We report here on the effects of a muscarinic compound with potent activity in animal models predictive of antipsychotic activity. A number of muscarinic agonists were active in these models and one compound, PTAC ((5R,6R)-6-(3-propylthio-1,2,5-thiadiazol-4-yl)-1-azabicyclo[3,2,1]octane), was chosen for further studies (20).

Methods

The methodology for the various techniques involved in the studies described here have been previously published (20,21).

Results and Discussion

Selectivity and functional effects of PTAC for muscarinic receptors

In radioreceptor binding assays in cell lines transfected with muscarinic receptors, PTAC had high affinity for the five muscarinic receptor subtypes, but, in contrast, had relatively low affinity for a number of other neuronal receptors including DA receptors subtypes (20; Table 1). The functional effects of PTAC on generation of second messengers was determined using cell lines transfected with muscarinic receptor subtypes. PTAC was a potent partial agonist in cells with muscarinic M_2 and M_4 receptors, but in phosphoinositide-coupled M_1, M_3 and M_5 containing cells PTAC had little if any agonist activity (Table 1). However, it was a potent antagonist of agonist-induced effects in cells transfected with muscarinic M_1, M_3 and M_5 receptors. In rats, PTAC but did not produce parasympathomimetic effects such as salivation or tremor, but blocked muscarinic agonist induced salivation and tremor (20). Thus, PTAC is relatively free from cholinergic side effects and interactions with non-muscarinic receptors and, therefore, is an ideal muscarinic ligand to investigate the potential role of muscarinic receptors in animal models predictive of antipsychotic activity.

Table 1. Inhibition of radioreceptor binding as well as efficacy and potency of PTAC in cell lines transfected with muscarinic M_1-M_5 receptors

Receptor	Binding K_i, nM[a]	% effect of full agonist	Agonist, nM[b]	Antagonist IC_{50}, nM
M_1	0.6±0.2	<1	-	0.4
M_2	2.8±1.4	57	0.6	-
M_3	0.2±0.02	<1	-	3.4
M_4	0.2±0.05	50	5	-
M_5	0.8±0.09	0	-	0.3

[a]Inhibition of binding was determined in CHO cell lines transfected with muscarinic receptor subtypes using [^{3}H]-N-methylscopolamine. [b]The ability of PTAC to stimulate formation of second messengers in cell lines was used to determine efficacy and the inhibition of full agonist-induced formation of second messengers was used to determine antagonism (20).

Inhibition of ex vivo binding by PTAC

After administration of PTAC to rats, the ex vivo binding of [^{3}H]-pirenzepine was inhibited in a dose related fashion (Fig. 1). Thus, PTAC or active metabolites readily penetrated into the brain after s.c. administration and potently inhibited ex vivo binding with an ED_{50} of 0.04 mg/kg s.c.

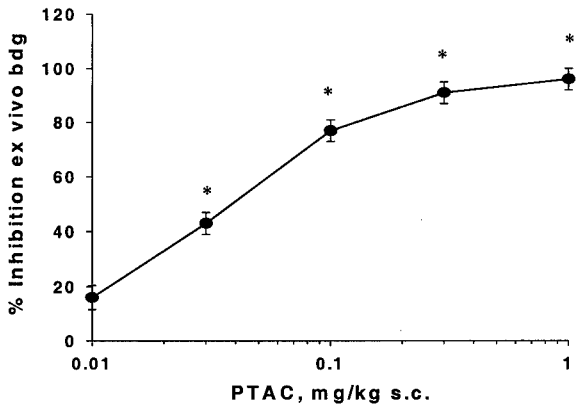

Fig. 1.
Dose-related inhibition of ex vivo binding of [^{3}H]-pirenzepine binding in cerebral cortex homogenates by PTAC. *=p<0.05

Effect of PTAC on conditioned avoidance responding and catalepsy

The ability of compounds to inhibit conditioned avoidance response is highly correlated with antipsychotic activity and blockade of DA D2 receptors (22). However, non-selective muscarinic agonists have also been shown to inhibit avoidance responding in this behavioral paradigm (12,23). The muscarinic agonist PTAC decreased conditioned avoidance responding in rats in a dose-related fashion to about 20% of control and the ED_{50} was 0.017 mg/kg s.c. (Fig. 2A). The PTAC-induced inhibition of conditioned avoidance responding was reversed by the muscarinic antagonist scopolamine, demonstrating that the effect was mediated by activation of muscarinic receptors (Fig. 2B). Dopamine antagonists such as haloperidol induce formation of abnormal

postures called catalepsy in animals and induction of catalepsy has been predictive of EPS in humans. In contrast, PTAC did not produce appreciable catalepsy up to doses 100-fold above the conditioned avoidance response ED_{50} dose (20).

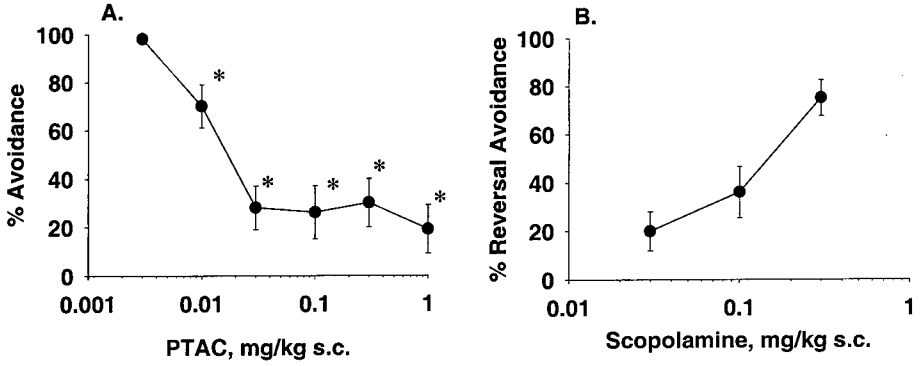

Fig. 2.
Dose-related inhibition of conditioned avoidance response in rats by PTAC (A.) and reversal of PTAC (0.1 mg/kg s.c.) induced inhibition of conditioned avoidance response by the muscarinic antagonist scopolamine (B.). *=p<0.05 Reversal data are from (20)

Inhibition of apomorphine-induced climbing

Since DA antagonists are used as therapy for schizophrenia and the disease has been hypothesized to be due to hyperactivity of DA neurons, blockade of DA agonist-induced behaviors has been used to predict antipsychotic activity. Inhibition of climbing behavior in mice induced by the DA agonist apomorphine is a behavioral model that has been used frequently to detect the DA antagonist activity of potential antipsychotic agents. Moreover, apomorphine is believed to produce climbing behavior by activation of mesolimbic DA neurons (24). Like DA antagonists, PTAC potently inhibited apomorphine (2 mg/kg s.c.) induced climbing behavior in a dose-dependent manner and with an ED_{50} of 0.005 mg/kg s.c. (Fig. 3). In addition, PTAC inhibited turning behavior induced by DA D1 or D2/D3 agonists in unilaterally 6-hydroxydopamine lesioned rats (data not shown).

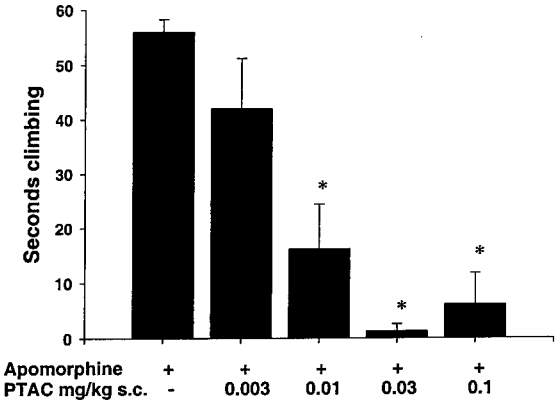

Fig. 3.
Dose-related inhibition of apomorphine (2 mg/kg s.c.) induced climbing behavior in mice by PTAC. Data are from (20). *=p<0.005

Inhibition of d-amphetamine-induced Fos expression

The protein Fos is the product of the immediate early gene cFos which is increased upon activation of many neurons and is involved in the regulation of gene expression. For example, typical and atypical antipsychotic agents increase Fos expression in several brain regions and regional induction of Fos expression has been used to map the areas of activation of these agents (25). In contrast, PTAC (0.1 mg/kg s.c.) alone did not increase Fos expression in the nucleus accumbens (Table 2), but PTAC (0.01-0.1 mg/kg s.c.) completely blocked the 3 fold increase in Fos expression induced by the DA releasing agent d-amphetamine (2.5 mg/kg s.c.). Thus, PTAC modulates the effects of dopaminomimetics in the mesolimbic pathway which is involved in the reinforcing properties of DA and probably also in schizophrenia.

Table 2. Inhibition of d-amphetamine (2.5 mg/kg s.c.)-induced Fos expression in nucleus accumbens of rat by PTAC (0.01 or 0.1 mg/kg s.c.).

Treatment	PTAC dose mg/kg s.c.	Fos positive nuclei/section
Saline	-	3.67±0.33
PTAC	0.1	5.33±1.77
d-Amphetamine	-	13.00±2.20*
d-Amphetamine + PTAC	0.01	5.00±0.84#
d-Amphetamine + PTAC	0.1	5.20±1.39#

Data are from (20). *=$p<0.05$ vs control; #=$p<0.05$ vs d-amphetamine alone

Electrophysiological studies on DA neurons

Electrophysiological studies investigating the number of spontaneously firing DA cells in VTA (A10) and substantia nigra (A9) brain regions have been used to evaluate antipsychotic agents (26,27). Dopamine antagonist antipsychotic drugs do not inhibit the firing of DA cells after acute administration, but may actually increase the firing rate of DA cells due to autoreceptor blockade. After chronic administration of DA antagonists, the long term activation depletes electrical gradients in the neurons and induces a state of depolarization inactivation (26). Therefore, chronic administration of typical antipsychotic drugs decrease the number of spontaneously firing DA cells in the VTA (A10) and substantia nigra (A9) midbrain areas. However, chronic administration of atypical antipsychotic agents like clozapine and olanzapine decrease the number of spontaneously firing DA cells only in the A10 tract thought to be involved in schizophrenia, but do not inhibit the motoric A9 tracts which may account for their lack of EPS (27). Chronic administration of PTAC (0.5 mg/kg/day s.c. in osmotic minipumps) to rats for 21 days reduced the number of actively firing DA cells in the A10 tract, but had little effect in the motoric A9 tract (Fig. 4A). In addition, acute administration of PTAC (0.1-500 μg/kg i.v.) inhibited the firing of DA cells in the A10 tract (ED_{50} 0.3 μg/kg i.v.) but was much less potent on A9 DA cells (ED50 = 100 μg/kg i.v., Fig. 4B). Thus, chronically administered PTAC had an effect similar to atypical antipsychotic agents, but uniquely also selectively inhibited A10 firing upon acute administration, suggesting potentially a rapid onset of action.

Alteration of tissue DA metabolite concentration

Dopamine antagonists produce large increases in tissue concentrations of the DA metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC), in DA-rich brain areas due to DA D2 autoreceptor blockade. Since PTAC blocked DA agonist-induced behavioral effects, the effect of PTAC and

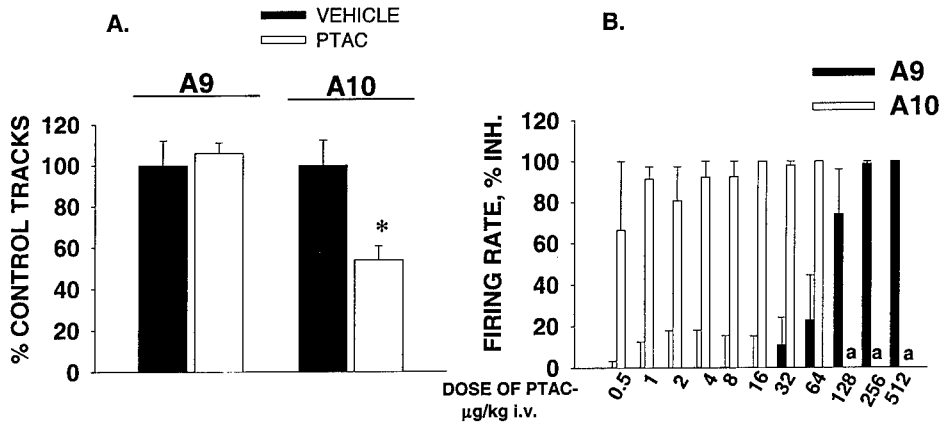

Fig. 4.
Effect of PTAC after chronic administration on the number of spontaneously firing DA cells/track (A.) and after acute administration on firing rate of DA cells in the A9 and A10 brain regions (B.). PTAC (0.5 mg/kg/day) was chronically administered s.c. in osmotic minipumps for 21 days. *=p<0.05 a= doses not tested. Data are from (20).

haloperidol on tissue concentrations of DOPAC in the DA projection areas neostriatum and nucleus accumbens was compared. Haloperidol increased the concentrations of DOPAC over 3 fold in both areas, but PTAC (0.01-1 mg/kg s.c.) did not appreciably alter DOPAC concentrations (Fig. 5). Thus, PTAC or its metabolites do not directly block DA receptors as is found with DA antagonists.

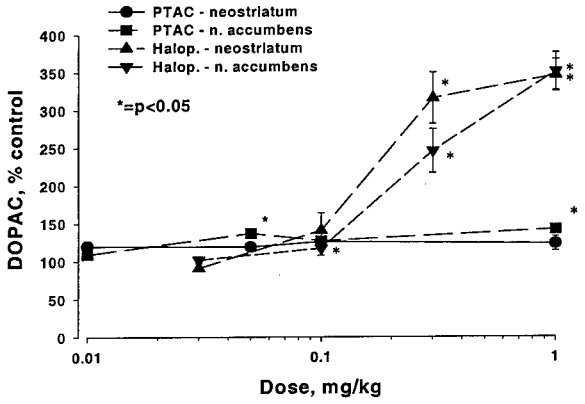

Fig. 5.
Effect of PTAC and haloperidol (Halop.) on DOPAC concentrations in the neostriatum and nucleus accumbens of rat. *=p<0.05

The data summarized here demonstrate that PTAC has potent and dose-dependent antipsychotic-like activity in a number of animal models predictive of antipsychotic activity. For example, PTAC inhibited conditioned avoidance responding, a behavioral model in which all antipsychotics are active (22). Furthermore, PTAC did not induce catalepsy as seen with many antipsychotic drugs, suggesting that PTAC would not produce EPS in humans. Even though PTAC does not have apparent affinity for DA receptors, it blocked DA agonist and dopaminomimetic-induced activity. For example, PTAC blocked climbing behavior induced by the DA agonist apomorphine.

Furthermore, it completely and potently blocked Fos expression induced by the DA releaser d-amphetamine. A potential explanation of the blockade of DA activities by PTAC is that PTAC modulates the postsynaptic response in mesolimbic pathways via interaction with muscarinic receptors either on the postsynaptic cells or downstream from the synapse. Moreover, the occupancy of muscarinic receptors as measured by inhibition of ex vivo binding was in the same general dose range as inhibition of conditioned avoidance responding and apomorphine-induced climbing, suggesting the activity in the behavioral models is mediated by muscarinic receptors.

Similar to clozapine and olanzapine, chronic administration of PTAC selectively decreased the number of spontaneously firing DA cells in the A10 tract, but had little effect in the A9 region. However, PTAC also potently inhibited the firing of the A10 DA cell after acute administration. Since PTAC acts acutely as well as chronically and does not block the DA autoreceptor, the chronic effect probably is not due to depolarization inactivation. The laterodorsal tegmental nucleus (C6) and pedunculopontine nucleus (C5) areas contain concentrations of cholinergic cell bodies whose axons project to the A9 and A10 DA areas, and presumably may alter the activity of the DA neurons by releasing acetylcholine onto muscarinic receptors on the A9 and A10 cell bodies (10). We speculate that PTAC may decrease the firing of the DA neurons by acting at muscarinic autoreceptors on the laterodorsal tegmental nucleus to inhibit this nucleus and remove excitatory drive or by directly interacting with potential inhibitory muscarinic heteroreceptors on DA cell bodies in the VTA.

These data demonstrate that PTAC has complex, multiple effects on DA neurons including inhibition of firing and blockade of postsynaptic effects. The exact mechanism of the effects are unknown, but do appear to be mediated by muscarinic receptors since PTAC is highly selective for muscarinic receptors. In addition, the antipsychotic-like effects of PTAC are blocked by muscarinic antagonists, demonstrating the effects involve activation of muscarinic receptors. Moreover, since PTAC is an agonist only at cAMP-coupled M_2 and M_4 receptors, the effect could be mediated at the cellular level by inhibition of cAMP formation. For example, the mRNA for M_4 receptors is colocalized with the mRNA for DA D1 receptors on striatonigral GABA neurons of the direct pathway that regulate the firing of DA neurons (28). Activation of M_4 receptors and the subsequent decrease in cAMP may modulate the activity of these neurons.

In summary, PTAC inhibited conditioned avoidance responding, exhibited functional dopamine antagonism, and selectively reduced the number of spontaneously firing of A10 DA neurons. Thus, the effects of PTAC are similar to the activity of several atypical antipsychotics in these models, although the effects of PTAC are mediated by muscarinic receptors. Moreover, PTAC also preferentially inhibited firing of A10 DA neurons following acute administration, an effect not observed with typical or atypical antipsychotic agents. Therefore, these data suggest that muscarinic agonists like PTAC may represent a new class of antipsychotic agents and could be a novel approach to the pharmacotherapy of schizophrenia.

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SCH 57790: A NOVEL M_2 RECEPTOR SELECTIVE ANTAGONIST

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Summary

As a decrease in cholinergic neurons has been observed in Alzheimer's Disease (AD), therapeutic approaches to AD include inhibition of acetylcholinesterase to increase acetylcholine levels. Evidence suggests that acetylcholine release in the CNS is modulated by negative feedback via presynaptic M_2 receptors, blockade of which should provide another means of increasing acetylcholine release. Structure-activity studies of [4-(phenylsulfonyl)phenyl]methylpiperazines led to the synthesis of 4-cyclohexyl-α-[4-[[4-methoxyphenyl]sulfinyl]-phenyl]-1-piperazineacetonitrile. This compound, SCH 57790, binds to cloned human M_2 receptors expressed in CHO cells with an affinity of 2.78 nM; the affinity at M_1 receptors is 40-fold lower. SCH 57790 is an antagonist at M_2 receptors expressed in CHO cells, as the compound blocks the inhibition of adenylyl cyclase activity mediated by the muscarinic agonist oxotremorine. This compound should be useful in assessing the potential of M_2 receptor blockade for enhancement of cognition.

Key Words: SCH 57790, M_2 receptor antagonist, Alzheimer's disease, acetylcholine

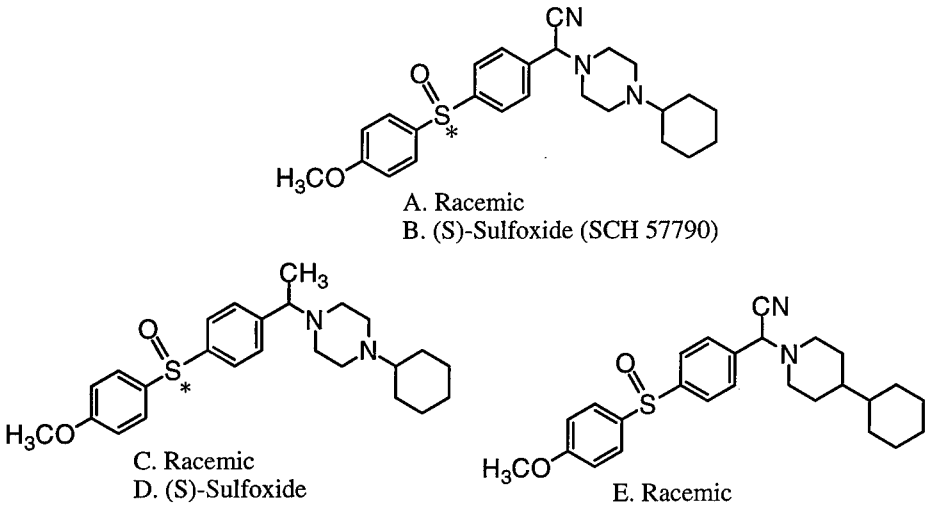
The neurotransmitter acetylcholine has been demonstrated to modulate learning and memory processes (1). Thus, the cholinergic system has been of interest in the study of cognitive disorders, including the neurodegenerative disorder known as Alzheimer's Disease (AD). Cholinergic neurons originating in the nucleus basalis of Meynert project to areas such as the cortex and hippocampus, brain regions which play a role in cognition (2,3). Lesioning these pathways in rodents produces a decrease in acetylcholine release and an impairment of memory-related task performance (4). This impairment can be reversed by cholinergic agonists (5). In AD, decreases in acetylcholinesterase levels and choline acetyltransferase activity have been reported (6,7). These observations indicate that the cholinergic system is compromised in AD and that drugs which stimulate cholinergic activity may provide relief from the neurodegenerative symptoms.

Currently, AD is treated with inhibitors of acetylcholinesterase, such as donepezil, which reduce the rate at which released acetylcholine is degraded for removal from the synapse (8). Another possible mechanism for augmenting central cholinergic activity is to facilitate acetylcholine release. Evidence suggests that in the CNS, presynaptic M_2 receptors act to inhibit acetylcholine release (9, 10). A selective M_2 antagonist which blocks this inhibition may therefore prove useful in the treatment of AD. SCH 57790 (4-cyclohexyl-α-[4-[4-methoxyphenyl]-(*S*)-sulfinyl]-phenyl]-1-piperazineacetonitrile) is a selective, high affinity M_2 antagonist. The interaction of this compound and a number of analogues (Fig. 1) with human muscarinic receptors is described.

Methods

The synthesis of SCH 57790 will be described elsewhere (Lowe, et al., manuscript in preparation). Human muscarinic receptor constructs were transfected into CHO-K1 cells by the calcium phosphate precipitation method. Colonies were selected for G418 resistance and tested for receptor expression by radioligand binding to *l*-quinuclidinyl [*phenyl*-4-^{3}H]benzilate ([^{3}H] QNB). Saturation experiments were performed using 5-2500 pM [^{3}H] QNB in 10 mM potassium phosphate buffer, pH 7.4, to determine the K_d of [^{3}H] QNB for each receptor subtype. Competition binding experiments were performed using 180 pM [^{3}H] QNB and 10-50 µg of membrane protein. All binding experiments were performed in the presence of 1% DMSO and 0.4% methylcellulose. Non-specific binding was defined by 0.5 µM atropine. After equilibrium was reached (120 minute incubation at room temperature) bound and free radioactivity were separated by filtration using Whatman GF-C filters. K_i values were derived from IC_{50} values using the Cheng-Prusoff equation (11).

Adenylyl cyclase assays were conducted using M_2 receptor-expressing CHO cells in suspension (approximately 250,000 cells/ 60 µl assay volume). To provide a stimulated baseline from which to observe oxotremorine-mediated inhibition, 3 µM forskolin was included in the assay. Phosphodiesterase activity was inhibited by 100 µM Ro-02-1724. Cells were pretreated with antagonist for 10 minutes prior to incubation with oxotremorine at 37°C for 5 minutes. A subsequent three minute 100°C incubation terminated the reaction and lysed the cells to release accumulated cAMP. Total cAMP in a 20 µl assay sample was quantified by a radioimmunoassay capable of detecting between 0.5 and 100 µM cAMP.



A. Racemic
B. (S)-Sulfoxide (SCH 57790)
C. Racemic
D. (S)-Sulfoxide
E. Racemic

Fig. 1
Chemical structures of SCH 57790 and analogues.

Results and Discussion

Fig. 2 shows SCH 57790 displacement of [^{3}H] QNB from human M_1-M_5 receptors. The muscarinic receptor binding affinities for SCH 57790 and its analogues are listed in Table 1. The K_i values derived from these competition experiments provide insights into the structure activity relationships of these compounds and muscarinic receptors. Substitution of a methyl group for the nitrile substituent confers higher affinity binding to muscarinic receptors, illustrated by compounds C and D. However, M_2 receptor selectivity *versus* M_1, M_3, and M_4 receptors is decreased by this substitution. The increased affinity for muscarinic receptors overall combined

with the loss in selectivity suggests that the methyl substituent interacts with amino acids that are common to all muscarinic receptor subtypes. The nitrile substituent may interact with residues that are specific to the M_2 receptor. The methyl compounds display the same stereoselectivity as the nitrile compounds; the (*S*)-sulfinyl enantiomer binds to muscarinic receptors with higher affinity than the racemic compound.

Compound E shows the ramifications of replacing the piperazine with a piperidine. This compound, which lacks the more basic nitrogen, is inactive at M_1 and M_2 receptors. It is postulated that this nitrogen forms an ionic interaction with the Asp 103 in the third transmembrane domain of the M_2 receptor. The aspartic acid in this position is common to all biogenic amine receptors and has been shown using receptor mutagenesis to be critical for agonist binding in muscarinic and other G-protein-coupled receptors (12, 13). The hypothesis that the piperazine nitrogen interacts with Asp 103 is further supported by results from receptor mutagenesis studies. Substitution of Asp 105 for Ala in the human M_1 receptor reduced the affinity of SCH 57790 by 14-fold.

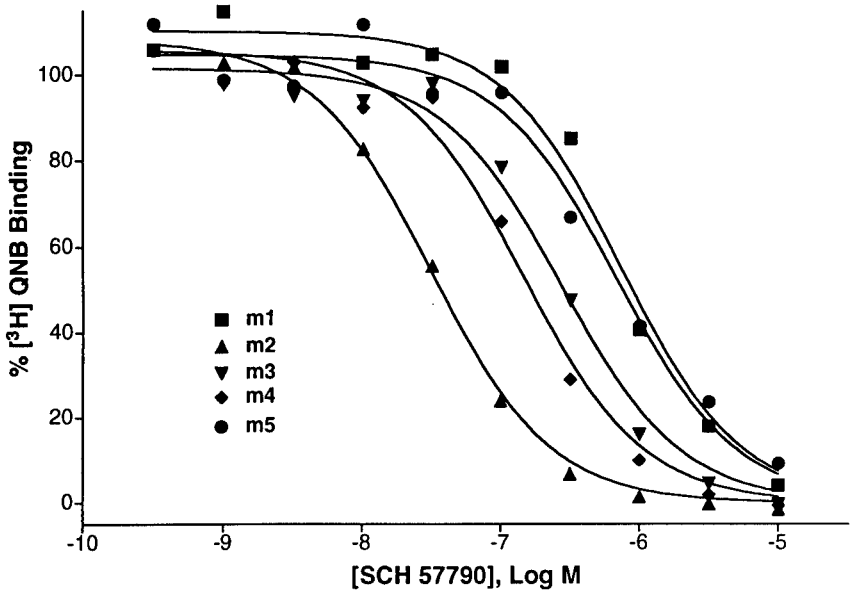


Fig. 2
Inhibition of [^{3}H] QNB binding by SCH 57790 at the five cloned muscarinic receptors.

TABLE 1

Affinities of SCH 57790 and Analogues at Human Muscarinic Receptors in CHO Cells.

	M_1 Ki, nM	M_2 Ki, nM	M_3 Ki, nM	M_4 Ki, nM	M_5 Ki, nM
A	114	9.1	80	38	NT
B	112	2.8	29	14	309
C	6.0	1.0	1.0	2.2	NT
D	4.7	0.3	0.4	0.7	NT
E	>500	>500	NT	NT	NT

NT= Not Tested.

Molecular models of SCH 57790 binding to the human M_2 receptor suggest that the sulfinyl moiety interacts with Trp 99 in TMIII (Fig 3). This interaction, which has been studied for M_1 receptors (14), is supported by the stereoselectivity observed for SCH 57790 and analogues. Substitution of Ala for Trp 99 in the M_2 receptor resulted in a 10-fold decrease in the affinity of SCH 57790. In addition, the models suggest that the distance between the piperazine nitrogen and the sulfoxide is 9 Å, consistent with the distance between Trp 99 and Asp103, which represents one helical turn.

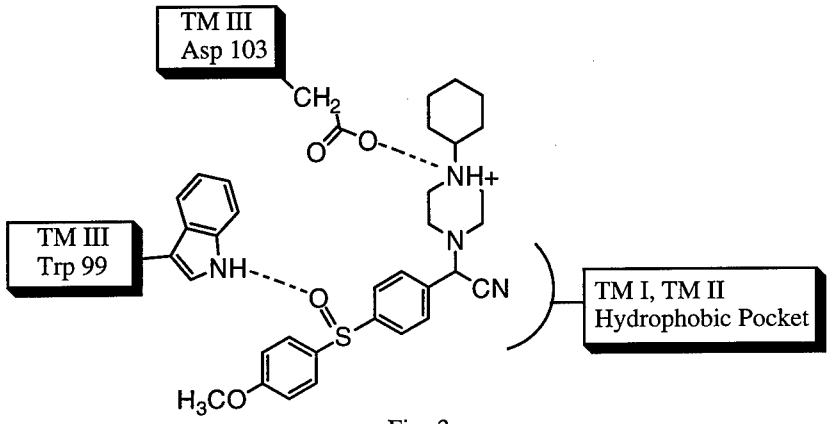


Fig. 3
Model of interactions between the human M_2 receptor and SCH 57790.

The muscarinic agonist oxotremorine-M produces a 30% maximal inhibition of cAMP produced by M_2-expressing CHO cells with an EC_{50} of 10 nM (Fig 4). SCH 57790 (30 nM) shifts the dose-response curve for oxotremorine to the right in a parallel fashion by 130-fold. In the presence of 100 nM SCH 57790, oxotremorine-M produces no inhibition of adenylyl cyclase.

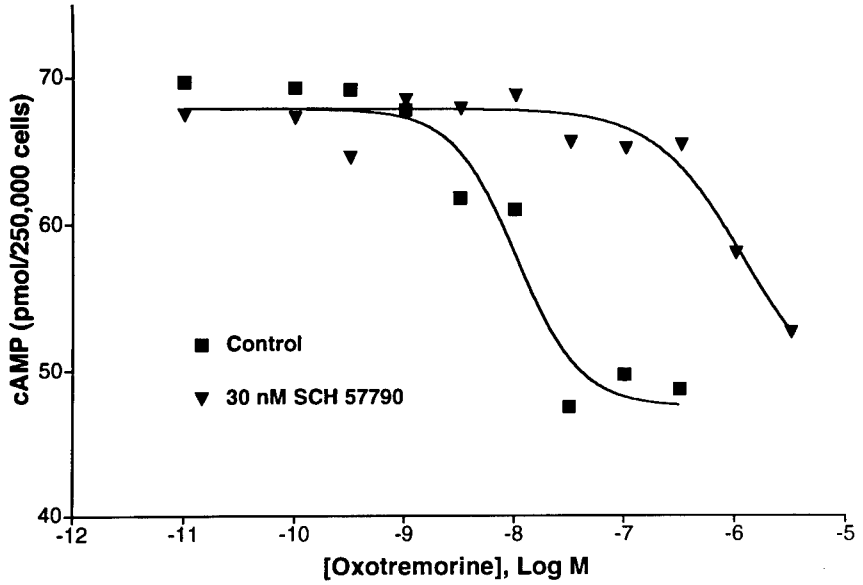


Fig. 4
Oxotremorine-mediated inhibition of forskolin-stimulated adenylyl cyclase activity.

Conclusion

Muscarinic receptors are G-protein coupled receptors for which mutagenesis studies have provided insights into structural moieties required for agonist and antagonist binding. The M_2 selective compound, SCH 57790 and related sulfinyl piperazine analogues provide new tools with which to study muscarinic receptor binding interactions. Among the [4-(phenylsulfonyl) phenyl]methylpiperazines that have been tested for muscarinic receptor affinity and M_2 receptor selectivity, the highest observed affinity is obtained when a methyl group is located between the phenyl and piperazine rings. Substituting a nitrile for the methyl in this position decreases the affinity at all muscarinic receptors, but the decrease is more pronounced for M_1 than M_2 receptors, particularly for the (*S*)-sulfinyl enantiomer. This enantiomer, SCH 57790, binds to M_2 receptors with a Ki of 2.8 nM and is forty-fold selective for the M_2 over the M_1 receptor. SCH 57790 has a basic nitrogen which appears to interact with Asp 103 in TMIII of the M_2 receptor. The sulfinyl group of SCH 57790 is postulated to interact with Trp 99. Further site directed mutagenesis studies will be required to elucidate additional interactions with the M_2 receptor and to determine the key residues which confer selectivity over other muscarinic subtypes.

SCH 57790 is an antagonist at M_2 receptors, demonstrated by the compound's ability to attenuate oxotremorine-mediated inhibition of adenylyl cyclase activity in CHO cells. This high affinity, M_2 selective compound may provide a means for increasing synaptic levels of acetylcholine by selectively blocking presynaptic muscarinic autoreceptors in the CNS. Future studies with this compound in animal models of cognition will demonstrate whether this mechanism of increasing acetylcholine release will enhance learning and memory functions.

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CHOLINERGIC FACILITATION OF TRACE EYEBLINK CONDITIONING IN AGING RABBITS

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Summary

The hippocampus is importantly involved in learning and memory, and is severely impacted by aging. In *in vitro* hippocampal slices, both the post-burst afterhyperpolarization (AHP) and spike-frequency accommodation are reduced in hippocampal pyramidal neurons after hippocampally-dependent trace eyeblink conditioning, indications of increased cellular excitability. The AHP results from the activation of outward potassium currents, including sI_{AHP} and muscarine-sensitive I_M. The AHP is significantly increased in aging hippocampal neurons, potentially contributing to age-associated learning deficits. Compounds which reduce the AHP and spike-frequency accommodation could facilitate learning in normal aging or in age-associated dementias such as Alzheimer's disease. The cholinesterase inhibitor metrifonate enhances trace eyeblink conditioning by aging rabbits and reduces the AHP and accommodation in hippocampal CA1 neurons in a dose-dependent manner. These reductions are mediated by muscarinic cholinergic transmission as they are blocked by atropine. Hippocampal neurons from metrifonate treated but behaviorally naive rabbits were more excitable and not desensitized to the effects of metrifonate since the AHP and accommodation were further reduced when metrifonate was bath applied to the neurons. These observations suggest that the facilitating effect of chronic metrifonate on acquisition of hippocampally dependent tasks is mediated at least partially by increasing the baseline excitability of CA1 pyramidal neurons. The issue of whether learning can be facilitated with muscarinic cholinergic agonists, in addition to cholinesterase inhibitors, was addressed by training aging rabbits during intravenous treatment with the M_1 agonist CI1017. A dose-dependent enhancement of acquisition was observed, with rabbits receiving 1.0 or 5.0 mg/ml CI1017 showing comparably improved learning rates as those receiving 0.5 mg/ml or vehicle. Sympathetic side effects, mainly excess salivation, were seen with the 5.0 mg/ml dose. Post-training evaluations suggested that the effective doses of CI1017 were enhancing responsivity to the tone conditioned stimulus. These studies suggest that muscarinic cholinergic neurotransmission is importantly involved in associative learning; that learning in aging animals may be facilitated by enhancing cholinergic transmission; and that the facilitation may be mediated through actions on hippocampal neurons.

Key Words: acetylcholine, afterhyperpolarization, aging, hippocampus, learning

The hippocampus is a critical neural system in learning which is very much affected by the aging process. Hippocampal lesions in humans and animals cause severe deficits in the ability to transfer information from short- to long-term stores and thus form new memories (1). We have adopted a hippocampally-dependent trace paradigm to analyze hippocampal involvement in learning (2,3). In the trace paradigm a blank "trace" period intervenes between conditioned stimulus (CS) offset and unconditioned stimulus (US) onset, which forces the rabbit to form a very short term memory of the CS in order to successfully predict US onset and perform conditioned responses timed properly to avoid the US. Trace eyeblink conditioning taps the hippocampal system's role in forming temporal associations and therefore this structure is necessary for learning this task (4,5).

Use of a hippocampally-mediated learning task is particularly relevant in studies of aging. Many studies have demonstrated structural (6), neurophysiological (7), and neurochemical (8,9) changes in the aging hippocampus, specifically to cholinergic systems. In addition to eyeblink conditioning, other hippocampally-dependent tasks such as spatial learning in rats (10) are also impaired in aged animals (7,11).

Eyeblink Conditioning : A Behavioral Model for Studying Learning Deficits in Aging

Eyeblink conditioning is used as a "model system" to analyze the neural substrates of learning (12,13,14). Eyeblink conditioning has many advantages for studying the neurobiology of learning deficits in both aged humans and animals (15,16). It is impaired in both older humans and animals (17,18). The changes across the lifespan of rabbits parallel those in humans (19), strengthening its validity as a model system. We have demonstrated that trace eyeblink conditioning is impaired in middle aged (24 mo) rabbits, and severely impaired by 30 mo of age (20). Additionally, aging rabbits are behaviorally heterogeneous, offering a subset of aging rabbits for study which are unable to learn even after extensive training.

Postsynaptic Outward Potassium Currents Regulate Neuronal Excitability in Learning

Both the afterhyperpolarization (AHP) which follows a burst of action potentials and spike frequency accommodation are reduced in CA1 and CA3 pyramidal neurons after hippocampally-dependent trace eyeblink conditioning (21,22). These reductions increase neuronal excitability and are well correlated with behavioral acquisition. Importantly, these excitability increases decay as expected if the hippocampus serves as an intermediate storage buffer in acquisition of new associations. These alterations are localized to the hippocampus after learning, as they occur in *in vitro* slices separated from their normal afferent and efferent connections (23). They are postsynaptic, as they are evoked by intracellular current injection and persist after block of sodium spike-dependent synaptic transmission (24).

An important conceptual issue regarding our experimental program should be addressed. When most neuroscientists think about how information is stored in neural networks during learning, changes located at the synapse are considered first. The description of how "Hebb synapses" might change during a hypothetical learning sequence (25) has inspired much work on model systems such as long term potentiation (26). But it should be pointed out that other mechanisms are available for altering synaptic efficacy, e.g., by modulation of excitability at the postsynaptic level (27). Adjustment of cellular excitability could amplify or attenuate synaptic changes occurring in distal dendrites (28), and affect neuronal firing output after learning. Possible functions of alterations in the afterhyperpolarization would be to control dendritic excitability, modulate neuronal gain or act as a temporal filter of information coming into the dendrites. This is especially relevant when considering cholinergic neurotransmission as involved in learning. Acetylcholine presumably acts as a neuromodulator by its action on slow outward potassium currents (27). Synaptic events, in this view of learning, produce post-synaptic changes with some degree of persistence, or "memory" of the associative event.

We have been quite successful in our efforts to define postsynaptic excitability changes in hippocampal neurons analyzed *in vitro* after eyeblink conditioning. We offer compelling evidence that calcium-mediated outward potassium currents are reduced in a conditioning-

specific fashion to increase hippocampal excitability in learning (20,21,29). Similar reductions in these or other outward potassium currents are well documented in invertebrate and mammalian learning models (30,31,32). The generality of our findings across vertebrate and invertebrate species suggests that postsynaptic modulation of outward potassium currents may be an important conserved mechanism used to mediate neuronal changes after learning (33).

Calcium-Activated Potassium Currents and Calcium Antagonists in Aging and Learning

The relevance of AHP reductions during learning in young adults to learning deficits in aging animals may be rather direct. Landfield and Pitler (34) first demonstrated that the AHP is prolonged in hippocampal CA1 neurons from aged rats. They suggested that this increase was a causative factor in learning impairments in aging. We found that both the AHP and spike frequency accommodation were increased in aging rabbit CA1 neurons, i.e., the neurons were less excitable (35).

One experimental approach of our own and of others used calcium channel blockers to address the hypothesis that an inability to reduce the AHP contributes to the learning deficits in aging animals. For example, elevation of plasma magnesium (a competitive inhibitor of calcium) improved reversal learning in both aged and young rats (36). Because of its ability to cross the blood brain barrier (37), the calcium antagonist nimodipine was tested in a variety of learning and behavioral tasks in aging mammals. Nimodipine markedly facilitated acquisition of the trace eyeblink conditioned response in aging rabbits (38,39,40) and humans (41); improved sensorimotor behaviors in aging rats (42); reversed open field deficits in aging rabbits (43); improved delayed matching-to-sample performance in aging primates (44); and improved spatial learning in aging rats (45,46).

The often dramatic behavioral effects of calcium antagonists in aging animals suggested that research focusing on compounds which reduced efflux through the slow calcium-activated potassium channel (sI_{AHP}) was a fruitful avenue for developing drugs that would facilitate learning in aging and dementia. Such an approach was based on our understanding of important cellular changes in young neurons during learning, and of cellular alterations in aging neurons that affected the learning process. Our observations that calcium channel antagonists increase hippocampal excitability *in vivo* (47) and reduce the AHP and decrease accommodation *in vitro* (35) in aging neurons at the same concentrations which facilitate learning in aging animals were important steps in determining cellular mechanisms of nimodipine's behavioral effects. A similar experimental approach should be fruitful in understanding mechanisms of action of cholinergic modulation of learning in aging brain.

Metrifonate Improves Associative Learning in Aging Rabbits

The hippocampus is a brain region which is importantly involved in mediating learning and memory (1,5) and has been shown to demonstrate alterations that correlate with the deficits in learning which occur during aging (48,49). Of special interest are the alterations that occur in cholinergic neurotransmitter systems that occur during aging and are especially prominent in Alzheimer's disease (8,50). This is the theoretical basis for one approach to the treatment of Alzheimer's disease in which reductions in cholinergic neurotransmission are compensated for by blocking cholinesterases which normally act to temporally limit the activity of acetylcholine. We have demonstrated that the cholinesterase blocker metrifonate improves hippocampally-dependent trace eyeblink conditioning in aging rabbits, a group in which learning is markedly impaired (20). In our first study, we showed that oral administration of metrifonate improved learning at doses of both 12 and 24 mg/kg, while 6 mg/kg was ineffective (51). The 24 mg/kg dose was somewhat less effective than the 12 mg dose, suggesting an inverted U dose/response curve. In addition, we began metrifonate treatment only one week prior to behavioral training but later determined that the asymptotic inhibition of cholinesterase in red blood cells did not occur until the third week of metrifonate treatment. A follow-up study, in which metrifonate pretreatment was begun three weeks prior to behavioral training, compared the effect of 12 and 24 mg/kg of metrifonate given

orally with 100 mM sodium citrate vehicle as a control in three groups of rabbits (52). The behavioral effect was stronger and less variable in the second experiment. Behavioral enhancement was clearly evident in the group learning curves by the fourth training session, had asymptoted after 15 training sessions, and was retained for 4 weeks after metrifonate administration was stopped. However, there was no difference in behavioral effectiveness between the 12 and 24 mg/kg doses in the second study. The metrifonate groups performed approximately twice as many conditioned responses as the control rabbits at the end of training.

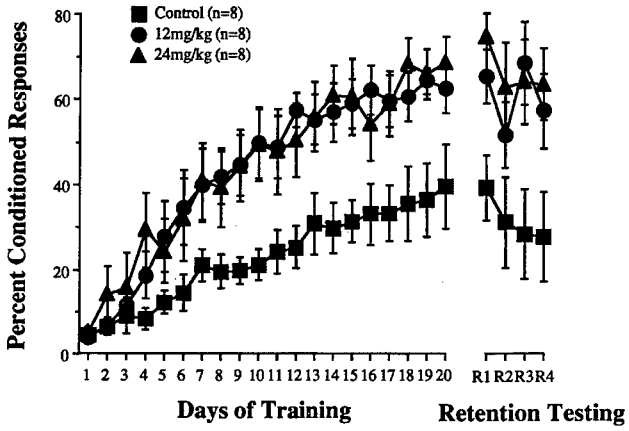

Fig. 1

Metrifonate enhanced acquisition early in training and improved retention of trace eyeblink conditioning for up to four weeks after dosing stopped in aging rabbits (reprinted from 52).

Cholinergic modulation of excitability in hippocampal CA1 neurons

Benardo and Prince (53,54) demonstrated that tonic cholinergic release occurred in the hippocampal slice, as muscarinic antagonists (such as atropine) decreased membrane resistance, via an increase in I_M (a tonic potassium current, sensitive to block by muscarine, that contributes both to the resting membrane potential and to the post-burst AHP). Anticholinesterases such as eserine (55) had opposite effects to atropine, mimicking the effects of bath (exogenous) application of carbachol or other ACh agonists. The net effect of cholinergic agonists was to increase the excitability of CA1 neurons (56). More specifically, exogenous carbachol or muscarine depolarized CA1 pyramidal cells and reduced both the AHP and spike-frequency accommodation (57), while specific nicotinic agonists such as dimethylphenylpiperazinium (DMPP) were without effect. Anticholinesterases strongly enhanced the effects of exogenous muscarinic agonists (57), but also when applied alone (i.e. without exogenous agonists) were still capable of significantly increasing CA1 excitability (58). All effects could typically be washed out in slices, indicating the reversability of receptor- or substrate-specific actions.

Metrifonate Increases Excitability in Young and Aging Hippocampal Neurons

One goal of our studies was to determine dose ranges of metrifonate (*in vitro*) that significantly reduce both the AHP and accommodation. We then compared these effects to those obtained after chronic treatment with metrifonate. Since enhanced excitability has been repeatedly demonstrated after learning by us (21,22,29,59) and others (60), and since CA1 excitability is severely reduced in aging (34,35), a link between these findings and between those with metrifonate should be readily observable. To this end, we examined the effect of metrifonate bath applied onto hippocampal slices from young and aging rabbits or chronically administered orally to aging rabbits before the slices were prepared (61). Metrifonate reduced the

afterhyperpolarization and spike frequency accommodation, i.e., increased neuronal excitability, in hippocampal neurons from both young and aging rabbits in a dose-dependent fashion in doses ranging from 10 - 200 μM. The reductions were mediated by muscarinic cholinergic transmission, as they were blocked by atropine. Aging rabbits chronically treated orally with metrifonate (12 mg/kg for 3 weeks) had significantly reduced spike frequency accommodation as compared to CA1 pyramidal neurons from vehicle-treated animals. Chronic metrifonate treatment did not desensitize the neurons to metrifonate, as bath application of metrifonate caused further reduction in both the AHP and accommodation in CA1 neurons. These data suggest that the facilitating effect of chronic metrifonate treatment on acquisition of trace eyeblink conditioning by aging subjects may be due at least partially to increased excitability of CA1 pyramidal neurons.

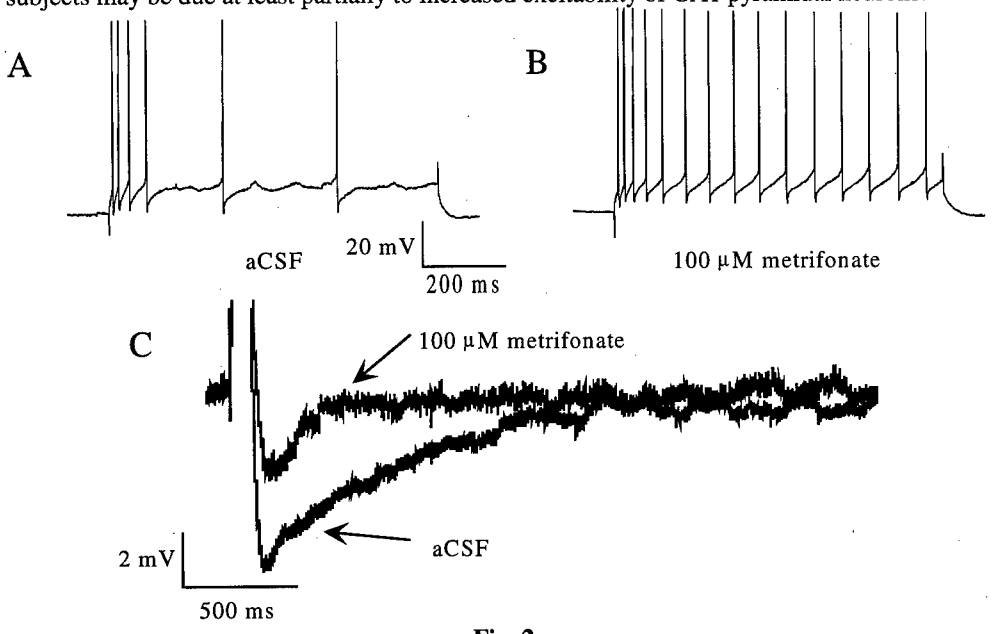

Fig. 2

100 μM metrifonate reduced spike frequency accommodation (A vs B) and the post-burst afterhyperpolarization (C) in CA1 pyramidal neurons from behaviorally naïve, untreated aging rabbits

The M_1 Agonist CI1017 Enhances Trace Eyeblink Conditioning in Aging Rabbits

A cholinesterase inhibitor such as metrifonate theoretically enhances cholinergic function by slowing the breakdown of neurotransmitter which is present at reduced levels in the aging or Alzheimer's disease affected brain. An alternative strategy is to activate muscarinic receptors directly with an agonist. The muscarinic receptor of most interest with respect to learning in aging brain is the M_1 type, which tend to be expressed most prominently in the brain and particularly in the hippocampus (62). The rationale for this approach, of course, is that the use of a selective M_1 agonist should activate the central receptors involved in learning and memory while causing minimal peripheral cholinergic side effects. We have therefore evaluated the effectiveness of CI1017 in hippocampally-dependent trace eyeblink conditioning. This compound has been identified as a relatively M_1-selective partial muscarinic agonist by cell metabolism, cell amplification, and second messenger assays (63), and has been shown to improve spatial learning in rodents and a continuous performance task in primates (64).

Drug delivery to aging (30-36 mo, avg wt 4.5 Kg) rabbits was by means of a chronically implanted subcutaneous venous access port. CI1017 was delivered at 2.7 ml/hr for 30 min before and during the daily trace eyeblink conditioning training sessions. Behavioral training procedures were identical to those used in previous studies, i.e., a 100 msec 6 KHz tone was paired with a

corneal airpuff after a 500 msec trace interval (20,38,51,52). Rabbits were given 80 paired conditioning trials each day. Three drug dose groups (5.0, 1.0, 0.5 mg/ml) plus vehicle were run. There were significantly more CRs seen in the 5.0 and 1.0 mg/ml groups of rabbits as compared to both the 0.5 and 0.0 mg/ml drug groups. The high (5.0 and 1.0) and low (0.5 and 0.0) drug groups were not different from each other, respectively. There were no differences in variables such as peak amplitude or latency of the conditioned response or of unconditioned response size between groups. The other interesting difference observed was that the generalization gradient (evaluated by systematically reducing the tone conditioned stimulus amplitude) was broader for the high drug groups as compared to the low dose groups when evaluated at the end of training during delay conditioning after the final session of trace conditioning. This indicated that the effective drug doses made the rabbits more responsive to the tone conditioned stimulus. Sympathetic side effects, primarily salivation, were prominent in the 5.0 mg/ml drug group. They were essentially nonexistent in the 1.0 mg/ml group, which had comparable behavioral enhancement to the higher dose 5 mg/ml group, and were also not present in the low dose rabbits. These data indicate that the M_1 agonist CI1017 is effective in facilitating learning of a hippocampally mediated associative learning task without causing unwanted peripheral sympathetic side effects. A comparison of the learning curves showed that CI1017 enhanced associative learning in a qualitatively and quantitatively similar fashion as did metrifonate (52).

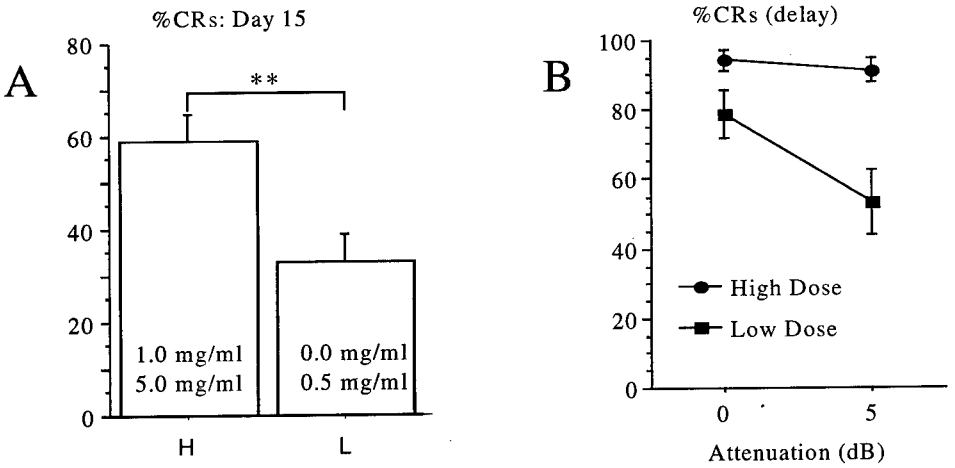

Fig. 3

A. CI1017 enhanced acquisition of trace eyeblink conditioning in aging animals at the 1.0 and 5.0 mg/ml doses. There was no difference in conditioned response probability on Day 1 but a highly significant increase in the higher dose animals on Day 15. **B.** Responsivity to the tone conditioned stimulus was also increased at the higher drug doses.

Comment

We have summarized the results of two separate studies which indicate that enhancing cholinergic neurotransmission with the cholinesterase inhibitor metrifonate is effective in facilitating associative learning in aging animals. This is not especially surprising, as cholinesterase inhibitors have been shown to be behaviorally effective in a variety of animal and human learning models. But the behavioral enhancements we showed are very substantial, have relatively little variability, last for several weeks following cessation of treatment, and occurred with no obvious side effects. This response profile suggests that metrifonate may be a quite useful compound in treating learning deficits associated with aging and/or Alzheimer's disease.

We have begun to address the mechanisms by which metrifonate may be working. Metrifonate enhances CA1 pyramidal neuron excitability in the hippocampus when bath applied to slices and after chronic treatment in a fashion that mimics the manner of administration in a

learning experiment. The reduced afterhyperpolarization and spike frequency accommodation are similar to those learning-specific changes observed in young and aging hippocampal neurons. Hippocampal neuron excitability has been observed to be reduced in aging animals, and suggested as one potential mechanism for aging-associated reductions in learning ability. Metrifonate may be facilitating learning by bringing hippocampal neuron excitability in aging animals into a range more like that of young animals. Our data also support the suggestion that one important index of the effectiveness of compounds in facilitating learning in aging mammals may be their ability to enhance the excitability of hippocampal and/or other temporal lobe system neurons.

Finally, we have demonstrated that the M_1 type muscarinic agonist CI1017 is effective in facilitating the acquisition of a well-controlled hippocampally-dependent associative learning task, trace eyeblink conditioning. In one sense, this is to be anticipated, given the striking learning facilitation we have demonstrated with metrifonate. But the demonstration of learning enhancements with muscarinic agonists has generally been difficult to demonstrate. The fact that we have observed comparable facilitation of learning with a muscarinic agonist as that we observed in the same age rabbits with a cholinesterase inhibitor, in the absence of side effects (for one of the effective doses), is heartening progress. Our pilot data indicates that CI1017 reliably raises CA1 pyramidal neuron excitability in young rabbits as metrifonate does.

Our studies indicate that hippocampally-dependent associative learning can be enhanced in aging rabbits by two different manipulations of the cholinergic system. Since eyeblink conditioning is hippocampally-dependent in the human (65,66) as it is the rabbit (2,3), it will be interesting to determine the effect of manipulations of the cholinergic system on this learning paradigm in the human. The implication of our work is that, if such a facilitation is seen in the human, we have an animal model available in which to fine tune pharmacological parameters to maximize the learning facilitation (dosing regimens, dose levels, age of effectiveness, etc.) and also one in which we can gain a cellular and molecular handle on how the compound(s) are working. This is obviously an important step in the rational design of even better compounds for reversing learning and memory deficits in aging.

Acknowledgements

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MUSCARINIC-MEDIATED ANALGESIA

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Summary

Systemic administration of cholinesterase inhibitors which cross the blood brain barrier have long been known to produce analgesia and enhance analgesia from opiates. A major site of analgesic action of cholinergic agents is the spinal cord. Muscarinic receptors are concentrated in the superficial layers of the dorsal horn of the spinal cord, an area of noxious sensory processing, and these reflect innervation primarily from cholinergic neurons with cell bodies deep in the neck of the dorsal horn. Spinal injection of cholinergic agonists results in analgesia which primarily reflects muscarinic receptor activation. Analgesia occurs in animal models of acute noxious stimulation and of chronic hypersensitivity pain. Although no cholinergic agonists have been tested for safety in humans, the cholinesterase inhibitor, neostigmine, has undergone such testing, and produces analgesia to experimental, acute postoperative, and chronic pain. Thus, muscarinic cholinergic agonists and cholinesterase inhibitors hold promise as non-opiate agents for the treatment of moderate to severe acute and chronic pain.

Key Words: analgesia, muscarinic receptors, spinal cord, acetylcholine

Fortunately, most pain is easily treated with traditional drugs (nonsteroidal anti-inflammatory drugs and opiates) by simple routes (oral). Description of opiate receptors in the spinal cord which inhibit transmission of nociceptive information led rapidly to spinal and epidural administration of opiates for analgesia. Compared to systemic injection, these invasive routes of administration allowed for profound pain relief with much smaller doses of drug, and in most cases fewer side effects. Side effects such as potentially catastrophic delayed respiratory depression have prompted further research to develop non-opiate analgesic agents with less worrisome side effects. Through these efforts, acetylcholine (ACh) and more than 25 other neurotransmitters have been identified which modulate pain processing in the spinal cord.

Cholinergic innervation of the dorsal spinal cord is primarily intrinsic, with cell bodies located in the deep dorsal horn (1). Cholinergic receptors are present in the superficial and deep dorsal horn of the spinal cord (1), areas of nociceptive information transmission and modulation. Both nicotinic and muscarinic agonists produce analgesia following intrathecal administration, reversible by specific nicotinic and muscarinic antagonists, respectively (2;3). Other studies suggest a pro-nociceptive action of nicotinic agonists, as suggested by presence of nicotinic ACh receptors (nAChRs) on primary afferents (4) and intrathecal nicotine-induced spinal glutamate release (5). Muscarinic agonists with different subtype selectivities produce behavioral analgesia in animals after systemic and spinal administration, although they produce several adverse side effects at approximately the same doses as those necessary for analgesia (2;6;7). For this reason we and others have examined other methods to activate ACh release rather than using direct agonists for analgesia.

ACh is released in response to physiologic and pharmacologic signals, especially pain itself, opiate receptor activation in the brainstem, and α2-adrenergic receptor activation in the spinal cord (Figure 1). As discussed above, nicotinic receptor stimulation can produce analgesia in the spinal cord, both by direct mechanisms and indirectly by stimulation of norepinephrine (NE) and ACh release. This component is probably small, since most studies fail to demonstrate reversal of analgesia from spinal administration of nicotinic antagonists.

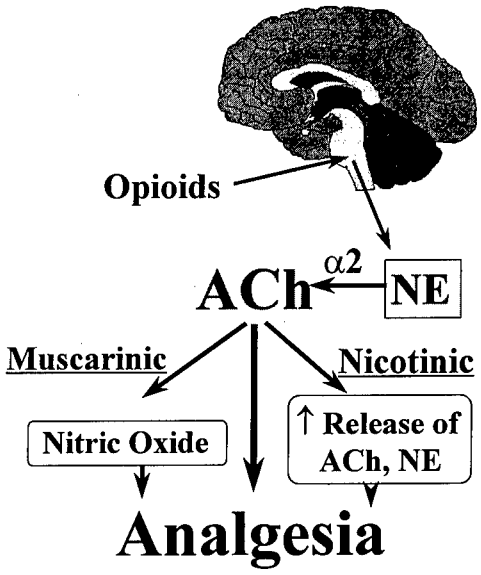

Fig. 1
Cholinergic mechanisms of analgesia

The role of spinally release ACh and subsequent muscarinic receptor activation in analgesia from systemic opioids is supported by studies utilizing a variety of experimental approaches. In rats, IV morphine produces a dose-dependent increase in latency to paw withdrawal from a noxious heat stimulus, and this effect is enhanced in a synergistic manner by spinal injection of the cholinesterase inhibitor, neostigmine (8). In humans, IV alfentanil produces an increase in cerebrospinal fluid (CSF) concentrations of ACh and a dose-dependent analgesia, and both of these effects were enhanced by spinal injection of neostigmine (9). These functional studies are supported by others measuring neurotransmitter concentrations from microdialysates from the spinal cord. For example, IV fentanyl or morphine administration in anesthetized sheep is associated with a dose-dependent increase in ACh and NE in dorsal, but not ventral horn microdialysates (10). Increases in ACh and NE are inhibited by IV naloxone, demonstrating an action on opioid receptors, and by cervical spinal cord transection, demonstrating an opioid-induced activation of descending spinal pathways. Increase in ACh is inhibited by spinal injection of idazoxan.

Other data support the role of spinal ACh in analgesia from spinally administered α2-adrenergic agonists. In sheep, spinal clonidine and dexmedetomidine produce dose-dependent antinociception to noxious mechanical stimuli, and both are similarly potentiated by spinal neostigmine (5-6 fold reduction in ED50) (11). Similarly, spinal neostigmine potentiates analgesia from epidural clonidine in humans (12). In anesthetized sheep, spinal injection of clonidine and dexmedetomidine increases ACh concentrations in dorsal horn, but not ventral horn microdialysates (13), and in CSF (14). In other studies in humans, spinal injection of clonidine increases ACh in CSF, whereas IV injection of clonidine or dexmedetomidine do not (15;16).

Several lines of evidence support a role for nitric oxide (NO) in the analgesic mechanism of spinal cholinergic analgesia. Neostigmine-induced enhancement of clonidine antinociception is reversed by spinal pretreatment with a NOS inhibitor (17). Antinociception from α2-adrenergic agonists alone is enhanced by spinal L-arginine (but not D-arginine), but is unaffected by pretreatment with a NOS inhibitor. Lack of blockade of α2-adrenergic antinociception by a NOS inhibitor, but enhancement by L-arginine is consistent with lack of blockade by atropine,

but enhancement by neostigmine, is consistent with a small ACh → NO component in normal animals. In anesthetized sheep, IV morphine increases NE, ACh, and nitrite, a stable metabolite of NO in dorsal horn microdialysates, and these increases are inhibited by IV naloxone or cervical spinal cord transection (18). Increases in ACh and nitrite are blocked by spinal idazoxan, and increase in nitrite is blocked by spinal NMLA, a NOS inhibitor. These data are consistent with the proposed spinal cascade activated by systemic opioids.

Pain itself may stimulate descending noradrenergic neurotransmission, leading to enhanced efficacy of spinally administered cholinesterase inhibitors. In studies in sheep, noxious electrical stimulation under general anesthesia results in an increase in CSF concentrations of NE and ACh which are blocked by cervical spinal cord transection, indicating activation of descending inhibition by noxious stimulation (19). This hypothesis of pain-induced activation of spinal ACh release is further supported by functional studies, in which spinal neostigmine in sheep produces antinociception which is greatest in the first day after laminectomy surgery, less the following day, and absent 5 days after surgery (20). This may be relevant to postoperative analgesia, since analgesic potency of spinal injection of neostigmine is greater in women after surgery than in volunteers tested with experimental noxious stimuli (21;22).

As noted above, spinal atropine has no effect on antinociception from spinal α2-adrenergic agonists in normal animals. To examine the altered pharmacology which could occur under conditions of chronic pain and hypersensitivity, we studied animals with L5 and L6 spinal nerve ligation. This model yields allodynia (perception of pain to a normally innocuous stimulus) to mechanical stimulation, which is stable over many weeks (23). In such animals, spinal clonidine produces an anti-allodynic effect which is completely reversed by spinal atropine and approximately 50% reversed by spinal hexamethonium or mecamylamine (24). Clonidine produces a concentration-dependent increase in nitrite in spinal cord tissue from these animals which is reduced in a dose-dependent manner by atropine and mecamylamine (25).

These data suggest that, following nerve injury, spinal α2-adrenergic agonists rely heavily on cholinergic receptor activation and resultant NO synthesis. In normal rats and sheep, antinociception to a mechanical or thermal stimulus from spinal injection of clonidine is enhanced by neostigmine, and this enhancement is eliminated by NOS inhibitors (14;26). However, antinociception from clonidine itself is unaffected by cholinergic antagonists or NOS inhibitors. We have argued (14) that this reflects a small contribution in the normal state by cholinergic - NO mechanisms, such that its amplification can enhance the effect of α2-adrenergic agonists, but its antagonism has little effect (Figure 2: normal). In contrast, this cholinergic - NO component is essential to the sustained/enhanced effect of α2-adrenergic agonists following nerve injury (Figure 2: neuropathic).

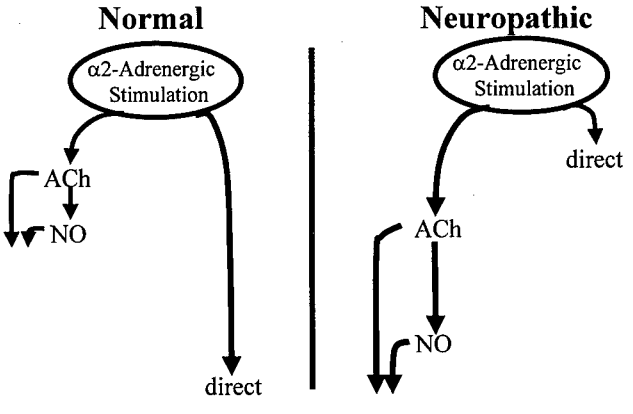

Fig 2.
Pharmacology of ACh Analgesia in Normal and Neuropathic States

In addition to producing analgesia, spinally administered muscarinic agents also affect hemodynamic control. Spinal neostigmine counteracts hypotension from spinal bupivacaine in rats (27). Since drug distribution and action can vary with the size of the spinal cord, we tested the effects of spinal neostigmine alone and with bupivacaine in conscious sheep (28). Neostigmine alone increased mean arterial blood pressure by 10%. Compared with spinal bupivacaine alone, addition of neostigmine resulted in hypotension of slower onset (15 vs 5 min) and smaller magnitude (-18% vs -37%). Addition of neostigmine did not affect height of sensory block from spinal bupivacaine. These data agree with preliminary clinical reports that large doses of spinal neostigmine diminish, but do not abolish, hypotension from spinal bupivacaine in humans. To test the hypothesis that this effect of neostigmine reflects actions on sympathetic nervous system activity, rats with chronically implanted spinal catheters were anesthetized and sympathetic efferent activity recorded from the left greater splanchnic nerve (29). Spinal injection of neostigmine doubled splanchnic sympathetic nerve activity. In contrast, spinal bupivacaine reduced splanchnic sympathetic nerve activity by 65%. Spinal co-administration of bupivacaine and neostigmine did not alter significantly splanchnic sympathetic nerve activity. These effects were paralleled by effects of treatments on blood pressure. These results suggest that spinal cholinergic stimulation by neostigmine counteracts hypotension from spinal bupivacaine by activation of sympathetic activity, occurring despite the presumed axonal blockade following spinal anesthesia.

In summary, muscarinic mediated analgesia occurs primarily by an action in the spinal cord. Both M1 and M2 selective agonists produce analgesia by spinal injection, although adverse side effects would likely limit this approach in clinical practice. The pharmacologic control of spinal release of ACh suggests that a combination of spinal α2-adrenergic stimulation or systemic opiate administration and spinal injection of a cholinesterase inhibitor could greatly enhance analgesia from the former while limiting side effects from spinal cholinergic stimulation.

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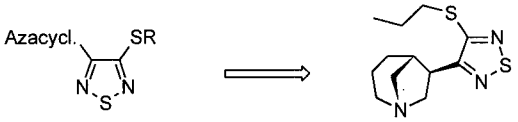
ABSTRACTS

1

MUSCARINIC AGONISTS WITH ANTIPSYCHOTIC-LIKE ACTIVITY. STRUCTURE ACTIVITY-RELATIONSHIPS OF 1,2,5-THIADIAZOLE ANALOGUES WITH FUNCTIOALLY DOPAMINE ANTAGONIST ACTIVITY.

P. Sauerberg[1]*, L. Jeppesen[1], P. H. Olesen[1], T. Rasmussen[1], M. D. B. Swedberg[1], M. J. Sheardown[1], A. Fink-Jensen[1], C. Thomsen[1], H. Thøgersen[1], J. S. Ward[2], D. O. Calligaro[2], N. W. DeLapp[2], F. P. Bymaster[2], H. E. Shannon[2]. [1]Health Care Discovery, Novo Nordisk A/S, Novo Nordisk Park, 2760 Måløv, Denmark and [2]Neuroscience Research, Lilly Research Laboratories, Indianapolis, Indiana 46285.

Muscarinic agonists were tested in two models indicative of clinical antipsychotic activity, conditioned avoidance responding (CAR) in rats and in inhibition of apomorphine climbing in mice. The standard muscarinic agonists oxotremorine and pilocarpine were both active in these tests, but showed little separation to cholinergic side effects. Structure activity-relationships (SAR) of the alkylthio-1,2,5-thiadiazole-azacyclic type muscarinic partial agonists showed the exo azabicyclo[3.2.1]octane analogue to give the optimal pharmacology compared to 1,2,5,6-tetrahydropyridine, quinuclidine, exo and endo azabicyclo[2.2.1]heptane and endo azabicyclo[3.2.1]octane analogues. SAR of the exo C_{1-6}alkylthio-1,2,5-thiadiazol-4-yl)-1-azabicyclo[3.2.1]octanes revealed the exo 6-(3-propyl/butylthio-1,2,5-thiadiazol-4-yl)-1-azabicyclo[3.2.1]octane analogues (**4a**, **4b** and **9a**, **9b**) to be the most potent antipsychotic agents with large separation to cholinergic side effects. The lack of enantiomeric selectivity suggested the pharmacophoric elements to be laying in the mirror plane of the compounds. A model explaining the potency differences of closely related compounds is offered. The data suggest that muscarinic agonists act as functional dopamine antagonists and that they could become a novel treatment of psychotic patients.



2

NEW POTENT MUSCARINIC 1,2,5-THIADIAZOLE-AZACYCLES AND DIMERS HEREOF.

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The observation that presynaptic neurons degenerate whereas the postsynaptic M1 receptors are preserved in Alzheimer's disease brain tissue led to the hypothesis, that enhancement or replacement of acetylcholine at these receptors would be beneficial in the treatment of Alzheimer's disease. In our effort to identify a potent M1 selective muscarinic agonist, we have developed a new series of 1,2,5-thiadiazole-azacycles **1**, having a phenylpropargyloxy/thio side chain (Fig.1). The compounds were initially tested in 2nd messenger assays using cell lines transfected with M1 or M2 receptor subtypes. A SAR around these highly efficacious M1 analogs is described. A more detailed biological profile is offered for selected compounds.
The series also includes some very potent dimers (twin-drugs) **2** (Fig.1), with a somewhat different, but still agonistic profile.

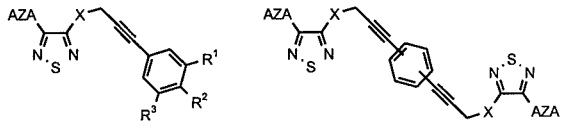

Fig.1 **1** X = O, S **2** X = O, S

3

BINDING AFFINITIES OF NEW DIMETHINDENE ANALOGUES AT FIVE MUSCARINIC RECEPTOR SUBTYPES

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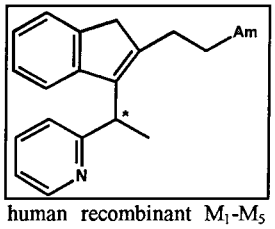

The (S)-(+)-enantiomer of the chiral drug dimethindene (**1**, Am=$N(CH_3)_2$) has been shown to be a potent and M_2-selective muscarinic antagonist (Pfaff et al., EJP 286: 229, 1995). Since there is a considerable interest in centrally active M_2-selective antagonists due to their diagnostic (PET imaging) and therapeutic potential in cognitive disorders, we have pharmacologically characterized a series of amino terminally modified dimethindene analogues. We hoped to alter the potency and selectivity in favour of the M_2-subtype and to decrease the affinity for H_1 receptors. Binding affinities (pK_i values) at muscarinic receptor subtypes were determined in radioligand competition studies at human recombinant M_1-M_5 receptors expressed in CHO cells using [^{3}H]N-methylscopolamine as radioligand. Histamine H_1 antagonism (pA_2 values) was determined in guinea pig ileum.

The results demonstrate that the antimuscarinic potency and subtype-selectivity of compounds **1-8** is highly (up to 51-fold) controlled by the size and shape of the cationic group **Am**. The H_1 affinity of compound **8** is 650-fold lower than that of dimethindene, whereas its M_2 selectivity is higher. According to the inverse stereoselectivity found for the enantiomers of dimethindene, we expect that resolution of racemic **8** into enantiomers results in a slight increase of the M_2 affinity and in a strong decrease of the H_1 affinity.

no.	Am	M_1	M_2	M_3	M_4	M_5	H_1
1	$N(CH_3)_2$	6.58	7.35	6.72	6.39	6.10	9.67
2	$N(c\text{-}C_4H_8)$	5.98	6.40	5.72	5.91	5.70	
3	$N(c\text{-}C_5H_{10})$	5.72	6.03	5.73	5.67	5.63	
4	$N((C_2H_4)_2O)$	5.36	5.84	5.56	5.38	5.57	
5	$N(C_2H_5)_2$	6.35	7.35	6.02	6.34	5.68	
6	$N(CH_3)(C_2H_4F)$	6.22	7.49	6.15	6.36	5.70	8.45
7	$N(CH_3)(iso\text{-}C_3H_7)$	6.09	6.84	5.74	6.00	5.66	
8	$N(iso\text{-}C_3H_7)_2$	6.07	7.30	5.64	6.14	5.71	6.86

Supp. by DFG.

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POTENT MUSCARINIC ANALGESICS DERIVED FROM EPIBATIDINE: ROLE OF THE M_4 RECEPTOR SUBTYPE

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Epibatidine, a neurotoxin isolated from the skin of *Epipedobates tricolor* is an efficacious antinociceptive agent with a potency 200 fold that of morphine. The toxicity of epibatidine, due to its nonspecificity for both peripheral and central nicotinic receptors, precludes its development as an analgesic. During the synthesis of epibatidine analogs we developed potent antinociceptive agents, typified by CMI-936 (2-exo{5-(3-methyl-1,2,4-oxadiazolyl)}-[2.2.1.]-7-azabicycloheptane) and CMI-1145 (2-exo{5-(3-amino-1,2,4-oxadiazolyl)}-[2.2.1.]-7-azabicycloheptane), whose antinociception, unlike that of epibatidine, is mediated via muscarinic receptors. Subsequently we used specific muscarinic toxins and antagonists to delineate the muscarinic receptor subtype involved in the antinociception evoked by these agents in female CD-1 mice. Thus, the antinociception produced by CMI-936 and CMI-1145 is substantially inhibited by 1) intrathecal injection of the specific muscarinic M_4 toxin MT-3, 2) intrathecally administered pertussis toxin, which inhibits the G proteins coupled to M_2 and M_4 receptors; and 3) subcutaneous injection of the M_2/M_4 muscarinic antagonist himbacine. Interestingly, the slight hypothermia and salivation elicited by these compounds was not blocked by any of the treatments above but was blocked by atropine. These results demonstrate that the antinociception elicited by these epibatidine analogs is mediated via muscarinic M_4 receptors located in the spinal cord. Compounds which specifically target the M_4 receptor may therefore be of substantial value as alternative analgesics to the opiates.

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SIMILARITIES IN ANTAGONIST AFFINITY PROFILES OF MUSCARINIC M_3 AND M_5 CHOLINOCEPTORS: PHARMACOLOGICAL DIFFICULTIES ASSOCIATED WITH IDENTIFYING A FUNCTIONAL M_5 RECEPTOR.

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The existence of five subtypes of muscarinic cholinoceptor is well established (see Eglen et al., 1996 for review). Difficulties in identifying an endogenous population of M_5 receptors, and thus ascribing a function to this gene product, stem largely from the lack of selective antagonists for this subtype. Moreover, the similarities in antagonist affinity profiles of the M_3 and M_5 subtypes, further complicate M_5 identification.

The present data highlights this similarity in antagonist affinity profiles, by providing affinity data in the human recombinant M_3 and M_5 receptors, expressed in Chinese hamster ovary cells, and comparing them with those of native M_3 receptors present in several smooth muscle preparations.

Antagonist	M_3	M_5	GPT	GPI	GPE	RUB
Pirenzepine	6.4	6.4	6.9	6.8	7.4	6.9
Methoctramine	6.8	6.3	5.9	6.1	6.0	6.2
AQ-RA 741	7.1	6.1	6.7	6.6	n/d	n/d
Himbacine	7.2	6.3	7.6	7.3	7.6	n/d
4-DAMP	8.8	8.6	8.9	8.9	9.0	9.4
p-F-HHSiD	7.6	6.6	7.2	7.6	8.2	7.5
Darifenacin	8.9	7.7	9.0	9.3	9.5	8.3
Zamifenacin	7.9	7.6	8.2	9.3	8.8	8.3

(M_3 and M_5 data are pK_B estimates from Watson et al., 1998, Br. J. Pharmacol 123, P268. Guinea-pig trachea (GPT), ileum (GPI), esophagus (GPE) and rat urinary bladder (RUB) data are pK_B estimates from Eglen et al., 1996, Pharmacol. Rev. 48, 531; n/d not determined).

Pirenzepine, methoctramine, 4-DAMP and zamifenacin demonstrated little or no selectivity between M_3 and M_5 subtypes. Characterization of the receptors in the smooth muscle preparations using only these antagonists generated antagonist affinity profiles which were ambiguous, correlating equally well with either the M_3 or the M_5 subtype. This suggests that, in the absence of *in situ* expression data, a functional M_5 receptor could be mistakenly classified as an M_3 receptor if the antagonist affinity profile is limited to these compound. Finally, these data identify himbacine, AQ-RA 741, p-F-HHSiD and darifenacin as defining ligands to differentiate M_3 from M_5 receptor-mediated responses. By extension, therefore, these antagonists also provide tools to identify a functional M_5 receptor.

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IONIC STRENGTH OF ASSAY BUFFERS INFLUENCES ANTAGONIST BINDING AFFINITY ESTIMATES AT MUSCARINIC M_1-M_5 CHOLINOCEPTORS .

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Antagonist binding affinity profiles are used to distinguish receptor subtypes and provide information regarding the selectivity of antagonists for one subtype over others. Therefore, experimental factors which influence antagonist binding affinity estimates may confound subtype identification and alter estimates of selectivity (Pedder et al., 1991, Br. J. Pharmacol., 103,1561). This study presents binding affinity data for several muscarinic receptor antagonists at the five human recombinant muscarinic receptor subtypes (M_1-M_5) expressed in Chinese hamster ovary cells. The affinities of these antagonists were evaluated using two buffers of different ionic strength: (i) Tris-EDTA buffer (50 mM Tris-HCl, 1 mM EDTA) and (ii) Tris-Krebs buffer (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 11 mM D-glucose, 25 mM Tris-HCl), both at pH 7.4 and 23°C.

Standard scintillation proximity assay (SPA, Amersham) methods were employed. The displacement curves were generated using 10 concentrations of test compounds and analyzed by iterative curve fitting to a four-parameter logistic equation. Atropine (1 μM) defined the non-specific binding.

Antagonist	M_1	M_2	M_3	M_4	M_5
Pirenzepine	8.2 (8.0)	6.5 (6.3)	6.9 (6.8)	7.4 (7.1)	7.2 (6.9)
Methoctramine	7.5 (6.7)	8.7 (7.7)	7.0 (6.1)	7.6 (7.0)	7.0 (6.3)
Tripitramine	8.9 (8.5)	9.9 (9.4)	7.8 (7.1)	8.5 (8.0)	7.9 (7.3)
Himbacine	6.8 (6.7)	7.7 (8.0)	6.9 (6.9)	7.5 (7.8)	6.1 (6.1)
PD 102807	5.6 (5.5)	5.4 (5.9)	6.1 (6.7)	7.1 (7.4)	5.3 (5.5)
MT3	6.7 (6.5)	5.9 (<5.4)	6.0 (<5.4)	8.1 (8.2)	6.0 (5.9)
Darifenacin	7.8 (7.8)	7.5 (7.0)	8.9 (8.8)	8.0 (7.7)	8.3 (8.0)
Zamifenacin	7.7 (7.6)	7.7 (7.2)	8.2 (7.9)	7.0 (6.9)	7.6 (7.3)

(Data are pK_i obtained using Tris-EDTA or Tris-Krebs buffer (data in parenthesis) and are the mean of 3-6 determinations, except MT3 where n=1-3; SEM < 5% of mean).

Affinity estimates at the muscarinic M_2 receptor subtype were consistently altered by the difference in buffer composition. Affinity estimates for methoctramine were most dramatically affected (10 fold), but differences were also observed for darifenacin and zamifenacin. These data indicate that buffer composition is an important factor in determining affinity and can alter the relative M_2/M_3 selectivity of antagonists.

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EXPRESSION OF RECOMBINANT m1-TOXIN1 IN YEAST.

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The most prevalent of the isotoxins of m1-toxin in the venom of the East African green mamba, *Dendroaspis angusticeps*, is m1-toxin1 (Carsi-Gabrenas and Potter, Neurosci. Abstr. 22, 1256, 1996). It binds selectively and irreversibly to m1 muscarinic receptors *in vitro* and *in vivo*, and it has proved to be a remarkably useful antagonist for identifying m1 receptors and for occluding m1 receptors so that m2-m5 receptors can be studied more easily (Purkerson and Potter, J. Pharmacol. 284, 707-713, 1998). However, the recovery of m1-toxin1 from a gram of lyophilized venom is only 200 μg, and the toxin is not stable to radioiodination. Therefore, the cDNA for m1-toxin1 was cloned using RACE techniques for use in an expression system (Potter, Krajewski and Dickerson, Life Sciences 60, 1205, 1997). The yeast *Pichia pastoris* were chosen because of their ability to grow to high densities in defined media, and for their ability to express high levels of recombinant proteins. The cDNA for m1-toxin1 was cloned into the expression vector pPIC3.5 downstream of the alcohol oxidase (AOX1) promotor. When the yeast were grown in media containing methanol, the promotor drove the expression of m1-toxin1, and the toxin was secreted into the media. The levels of m1-toxin1 produced have varied from 2-5 mg/liter of media, and the toxin can be purified directly from the media by cation-exchange chromatography. This expression system will be used to generate large quantities of recombinant m1-toxin1, and to express ^{3}H-m1-toxin when the yeast are grown in the presence of tritiated amino acids. In addition, site-directed mutagenesis will be used to produce mutant recombinant forms of m1-toxin1, to evaluate the amino acid residues that are responsible for the remarkable subtype selectivity and affinity of this toxin. Supported by AG 12976.

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CO-OPERATIVE POTENTIAL OF M_2 MUSCARINIC RECEPTORS

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The antagonists [^{3}H]quinuclidinylbenzilate (QNB) and *N*-[^{3}H]methylquinuclidinylbenzilate (NMQ) were examined for non-competitive effects at digitonin-solubilised muscarinic receptors purified from porcine atria and devoid of G protein. Binding was measured at equilibrium and 30 °C, as described previously [Wreggett and Wells (1995) *J. Biol. Chem. 270*, 22488].

Each radioligand revealed a single class of sites (log K_P = −9.20 ± 0.05, QNB; log K_P = −9.21 ± 0.15, NMQ), as measured at concentrations up to about 500 nM, but the apparent capacity for [^{3}H]NMQ was only 12% of that for [^{3}H]QNB in the same preparation. The affinity of [^{3}H]NMQ therefore was unmeasurable for 88% of the sites (*i.e.*, log K_P > −5.20). The relative capacity was unchanged when unlabelled NMQ (2 μM) was pre-incubated with the receptor and then removed by size-exclusion chromatography, suggesting that NMQ had no irreversible effect on the system. The specific binding of [^{3}H]QNB, at the near-saturating total concentration of 10 nM, was inhibited fully by unlabelled NMQ (n_H = 0.81 ± 0.10, log IC_{50} = −7.30 ± 0.13). If the inhibition is assumed to be competitive, the equilibrium dissociation constants (K_{Aj}) inferred for NMQ at 12% and 88% of the sites recognised by [^{3}H]QNB are 0.59 nM and 4.1 nM, respectively (log K_{A1} = −9.23 ± 1.00, log K_{A2} = −8.39 ± 0.10). Unlabelled NMQ therefore inhibited the binding of [^{3}H]QNB at the 88% of receptors that were inaccessible to radiolabelled NMQ (cf. log K_{A2} = −8.39 and log K_P > −5.20).

The non-competitive effects of unlabelled NMQ on the binding of [^{3}H]QNB point to co-operativity among interacting sites, and a similar phenomenon may account for the low capacity revealed by [^{3}H]NMQ. Previous studies have suggested that M_2 receptors are at least tetravalent, and the nucleotide-sensitive, multiple classes of sites revealed by agonists and antagonists can be attributed to co-operative interactions between successive equivalents of ligand [*e.g.*, Chidiac *et al.* (1997) *Biochemistry 36*, 7361]. The ratio of 1:8 defined by the capacities for [^{3}H]NMQ and [^{3}H]QNB implies at least eight interacting sites. (Supported by the Medical Research Council of Canada)

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THE INTERACTION OF OTENZEPAD (AF-DX 116) WITH ALLOSTERIC MODULATORS AND COMPETITIVE ANTAGONISTS AT A MUSCARINIC M_2 RECEPTOR

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Functional studies conducted in guinea-pig left atria, involving combination of otenzepad with the competitive antagonists N-methylscopolamine (NMS), dexetimide or atropine, or the allosteric modulators, C_7/3′-phth (heptane-1,7-bis(dimethyl-3′-phthalimidopropyl)ammonium bromide) or gallamine provided evidence that the action of otenzepad is exerted at an allosteric site on muscarinic M_2 receptors. Otenzepad, in combination with C_7/3′-phth or gallamine, gave concentration ratios close to additive and in agreement with theoretical predictions for combination of two allosteric modulators, whereas in combination with dexetimide or NMS, supra-additive concentration ratios were obtained. For either competitive antagonist in combination with otenzepad the degree of supra-additivity was more evident after 2 hr equilibration than after 40 min. When otenzepad was combined with atropine, no supra-additivity was observed with carbachol as the agonist, but was evident with acetylcholine. Otenzepad was also unable to fully inhibit [^{3}H]-NMS binding when the radioligand was employed at a concentration of ~ 100 × K_D. It is concluded that the action of otenzepad may be explained by an interaction at an allosteric site common to that of C_7/3′-phth and gallamine, and different from the site at which competitive antagonists bind.

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ASSESSMENT OF COOPERATIVITY FACTORS BY THE USE OF A [^{3}H]LIGAND FOR THE ALLOSTERIC SITE OF MUSCARINIC M_2 RECEPTORS

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Allosteric modulation of ligand binding to muscarinic receptors is commonly characterized by the cooperativity between the modulator at the allosteric site and a radioligand such as [^{3}H]N-methyl-scopolamine ([^{3}H]NMS) at the orthosteric site. The cooperativity factor α represents the ratio of the alloster binding affinity at the ligand occupied receptor and at the free receptor, respectively. Here, it was checked whether the novel radioalloster [^{3}H]dimethyl-W84 can be used to determine the cooperativity between the prototype allosteric modulator gallamine and NMS at muscarinic M_2 receptors (porcine cardiac membranes, 4mM Na_2HPO_4, 1mM KH_2PO_4, pH 7.4, 23°C; results are given as means ± S.E.M.). First, α was measured in the common way by equilibrium binding experiments using [^{3}H]NMS (at 0.2 and 1.6 nM, pK_D = 9.68 ± 0.10, n=8). Gallamine inhibited [^{3}H]NMS binding indicating negative cooperativity. The equilibrium dissociation constant of gallamine at the free receptor amounted to pK_x = 8.35 ± 0.09, the cooperativity factor between gallamine and NMS was α = 46 (n=5). Since cooperativity is reciprocal in nature, NMS should diminish the affinity of gallamine by the same factor. Applying the radioalloster [^{3}H]dimethyl-W84 (0.3 nM, pK_D = 8.89 ± 0.18, n=4) to label the allosteric site and increasing concentrations of gallamine to inhibit [^{3}H]dimethyl-W84 binding yielded a pK_i for gallamine at the free receptor of 8.27 ± 0.39 (n=5). In the presence of 1 µM NMS to occupy the orthosteric site gallamine displaced radioalloster binding (pK_D = 8.74 ± 0.09, n=7) with a pK_i of 6.60 ± 0.19 (n=3). The reduction of gallamine binding affinity by NMS corresponds to a cooperativity factor of α = 47.
In conclusion, independent of whether a radiolabeled orthosteric ligand or a radiolabeled allosteric ligand was used the same factor of cooperativity between gallamine and NMS was found. This result is in accordance with the cooperativity model.

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MODES OF ALLOSTERIC INTERACTIONS WITH MUSCARINIC M_2 RECEPTORS AS DEDUCED FROM BUFFER-DEPENDENT POTENCY SHIFTS

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The mode of allosteric interaction of the dichlorobenzyl substituted bispyridinium compound Duo3 with muscarinic M_2 receptors occupied by N-methylscopolamine (NMS) is known to differ from that of common site allosteric agents. It is one particular feature of Duo3 that its potency to retard [^{3}H]NMS-dissociation is almost the same under different assay conditions. In order to gain insight into whether the action of Duo3 at free M_2 receptors is characterized by the same particular mode as its interaction with NMS-occupied receptors, we measured the retarding effect of Duo3 on the association of [^{3}H]NMS to porcine cardiac M_2 receptors under two assay conditions: „Na,K,P_i“, 4 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.4, 23° C and „Mg,Tris,Cl,P_i“, 3 mM $MgHPO_4$, 50 mM Tris-HCl, pH 7.3, 37° C. The result (see table) was compared with the effect of Duo3 on [^{3}H]NMS-dissociation. A common site allosteric agent, the phthalimidomethyl substituted bispyridinium WDuo3, was included. EC_{50}: concentration in nM reducing the apparent rate constant by 50 %, n_H: Hill slope of the curve ($^{\#}$ different from unity, $p < 0.05$); each curve is based on n=12-20 kinetic measurements.

		Na,K,P_i	Mg,Tris,Cl,P_i	EC_{50}shift
Duo3	$EC_{50,ass}$ (n_H)	51 (-1.55$^{\#}$)	74 (-1.47$^{\#}$)	1.5
	$EC_{50,diss}$ (n_H)	450 (-1.59$^{\#}$)	1246 (-1.72$^{\#}$)	2.8
WDuo3	$EC_{50,ass}$ (n_H)	12 (-1.03)	359 (-1.04)	30
	$EC_{50,diss}$ (n_H)	10 (-0.95)	227 (-1.15)	23

The interaction of Duo3 with free M_2 receptors resembles its interaction with NMS-occupied receptors. With regard to both the steepness of the concentration-effect relationships and the buffer dependence of the effects, Duo3 is clearly divergent from WDuo3.

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PROBING THE OVERLAP OF THE SELECTIVE ANTAGONIST AF-DX 384 WITH THE ALLOSTERIC SITE OF MUSCARINIC M_2 RECEPTORS

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The interaction between the M_2-preferring antagonist AF-DX 384 and the allosteric agent W84 (hexane-1,6-bis[dimethyl-3-(phthalimidopropyl)-ammonium bromide]) as observed in contracting atria and in homogenates of guinea pig hearts suggested that the binding area of AF-DX 384 partially overlaps with the common allosteric site of M_2 receptors. Now, truncated derivatives of W84 were applied to find out whether the interference of AF-DX 384 with alloster action depends on the length of the W84 derivative. Effects on the dissociation of [^{3}H]AF-DX 384 were determined in guinea pig heart homogenates (3 mM $MgHPO_4$, 50 mM TrisHCl, pH 7.3, 23 °C). For comparison [^{3}H]N-methylscopolamine ([^{3}H]NMS) was applied which does not overlap with the allosteric site (buffer as above, 37 °C). Concentration-effect curves for the allosteric reduction of the apparent rate constant of dissociation k_{-1} were fitted with slopes of unity. For [^{3}H]NMS, curves approached $k_{-1} = 0$ %; for [^{3}H]AF-DX 384, the effects of the compounds were submaximal (minimum k_{-1}: 17-46 %). The inflection points of the curves, EC_{50}, may represent the affinity of the compounds to the ligand occupied receptors. Similar to W84 ($EC_{50,[3H]AF\text{-}DX}$ = 31 µM, $EC_{50,[3H]NMS}$ = 1.3 µM), its derivative with phthalimidomethyl cut off at one end was considerably less potent with [^{3}H]AF-DX 384 than with [^{3}H]NMS ($EC_{50,[3H]AF\text{-}DX}$ = 134 µM, $EC_{50,[3H]NMS}$ = 14µM). Unilateral shortening including one of the quaternary nitrogens made the potency to inhibit dissociation independent of the radioligands ($EC_{50,[3H]AF\text{-}DX}$ = 79 µM, $EC_{50,[3H]NMS}$ = 75 µM). Thus, with this extent of shortening the alloster appears to be devoid of the moiety that is hindered by AF-DX 384 from attaching to the allosteric site. The findings are compatible with the notion that AF-DX 384 utilizes about half of the W84 allosteric binding domain.

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ALLOSTERIC POTENTIATION OF THE EFFECTS OF MUSCARINIC AGONISTS

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It was found in earlier radioligand binding experiments on membranes of genetically engineered CHO cells that the affinities of muscarinic M_4 receptors for the agonists furmethide, oxotremorine-M and bethanechol were increased and those of the M_2 receptors were diminshed by the allosteric modulator brucine. The affinity for oxotremorine was little changed by brucine on the M_4 receptors and diminished on the M_2 receptors, and the affinities for furmethide were diminished by the allosteric modulator alcuronium on both the M_4 and the M_2 receptors (ref. 1). To see whether the observed allosterically induced changes of the affinities for muscarinic agonists are relevant functionally, we examined the effects of brucine and alcuronium on the inhibition of [^{3}H]acetylcholine release by presynaptic muscarinic autoreceptors. Experiments were performed on superfused electrically stimulated slices of rat striatum preloaded with [^{3}H] choline. The release of [^{3}H]acetylcholine was diminished by all four agonists tested (i.e., furmethide, oxotremorine-M, bethanechol and oxotremorine). Brucine enhanced the inhibitory effects of furmethide, oxotremorine-M and bethanechol and did not alter the effect of oxotremorine. Alcuronium diminished the inhibitory effect of furmethide. The direction of the observed effects of brucine and alcuronium on the inhibitory potencies of muscarinic agonists with regard to the release of [^{3}H]acetylcholine fully agreed with the effects of these modulators on the affinities of the M_4 receptors (but not of the M_2 receptors) for the investigated agonists. We conclude that (a) it is possible to enhance the functional effects of muscarinic receptor agonists by allosteric modulators, and (b) the presynaptic muscarinic receptors responsible for the inhibition of the release of acetylcholine in the striatum belong to the M_4 receptor subtype.

(1) Jakubík J., Bačáková L., El-Fakahany E.E., Tuček S.: Mol. Pharmacol. 52:172-179, 1997.

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CHARACTERIZATION OF THE SUBTYPE SELECTIVITY OF THE ALLOSTERIC MODULATOR, C_7/3-PHTH, AT CLONED MUSCARINIC ACETYLCHOLINE RECEPTORS.

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We investigated the selectivity of binding of the allosteric muscarinic acetylcholine receptor (mAChR) modulator, heptane-1,7-bis-(dimethyl-3′-phthalimidopropyl) ammonium bromide (C_7/3-phth), at the five human mAChR subtypes, stably expressed in Chinese hamster ovary cells. In equilibrium binding assays, increasing concentrations of C_7/3-phth resulted in a concentration-dependent inhibition of the specific binding of the radioligand [^{3}H]N-methylscopolamine ([^{3}H]NMS) at all receptor subtypes, with the highest affinity being observed at the M_2 receptor, and the lowest affinity at the M_5 receptor. An allosteric mode of interaction was indicated by the inability of C_7/3-phth to completely inhibit [^{3}H]NMS binding, particularly when a high radioligand concentration was employed. From these experiments, the rank order of negative cooperativity between C_7/3-phth and [^{3}H]NMS at the five mAChR subtypes was: $M_5 > M_2 > M_3 > M_4 \sim M_1$. In non-equilibrium, dissociation kinetic binding experiments, C_7/3-phth decelerated the dissociation of [^{3}H]NMS at all five mAChR subtypes in a concentration-dependent manner, supporting the notion of an allosteric mode of interaction. Analysis of the kinetic experiments yielded independent estimates of the affinity of C_7/3-phth for the occupied receptors, which were in good agreement with the values derived from the equilibrium binding assays.

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SEPARATE EPITOPES CONTRIBUTE TO GALLAMINE'S AFFINITIES FOR M_1 AND M_5 MUSCARINIC RECEPTORS

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Gallamine allosterically modulates the binding of classical muscarinic ligands with a potency order of M_2>M_1,M_4>M_3,M_5. Using chimeric receptors, we have demonstrated that the M_2/M_5, and probably also M_2/M_3, selectivity is due to an epitope in the third outer loop (o3) of the receptor[1]. Others have demonstrated the presence of another epitope in the o2 loop of the M_2 receptor, glu-asp-gly-glu (EDGE)[2]; when these residues in the M_2 subtype are replaced by corresponding residues from the M_1 receptor, leu-ala-gly-gln (LAGQ), the affinity of gallamine for the mutated M_2 receptor is reduced, compared to wild-type M_2. The apparent paradox that different epitopes are responsible for gallamine's higher affinity at M_2 is clarified by considering the sequences of all five subtypes in these two regions (bold letters indicate residues referred to in text):

	o2-region	o3-region		o2-region	o3-region
M_2	TV **EDGE** CY	IP **N** TV	M_2	TV **EDGE** CY	IP **N** TV
M_1	TV **LAGQ** CY	VP **E** TL	M_5	TV PL**DE** CQ	VP **V** TL
M_4	TV PDNH CF	IP D TV	M_3	TV PPGE CF	IP **K** TF

We have found that the mutation LAGQ→EDGE in the o2 region of M_1 produces an increase in affinity toward gallamine. The mutation DE→GQ in the o2 region of M_5 yields a decrease in affinity. In the o3 region, mutation of V→N in M_5 or K→N in M_3 results in an increased affinity. In M_2, the converse mutation of N→K decreases affinity toward gallamine; the N→E mutation yields a slight increase in affinity. Thus, we conclude that M_5 (and probably also M_3) derives affinity from gallamine's interaction with the o2 region, while M_1 (and probably M_4) derives affinity from an interaction with the o3 region. Gallamine appears to interact with both regions of the M_2 subtype.

[1]*Mol Pharmacol 44:583, 1993;* [2]*Leppik et al., Mol Pharmacol 45:983, 1994.* *Supported by R01 AG05214.*

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SITE-DIRECTED MUTAGENESIS OF mAChR DEMONSTRATES VTIL MOTIF PARTICIPATION IN AGONIST-INDUCED SEQUESTRATION AND G PROTEIN COUPLING.

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The rapid phase of desensitization for G protein coupled receptors involves uncoupling from the G protein and internalization or sequestration of the receptor. Through deletion mutations and the construction of chimeric proteins, several groups have found that multiple domains of the muscarinic cholinergic receptor (mAChR) are involved in sequestration and that some of these same domains participate in G protein coupling. In this study, we used site-directed mutagenesis to assess the importance in the sequestration process of the highly conserved VTIL motif at the third intracellular loop junction with the sixth transmembrane spanning domain in M2 mAChR. The effect of each mutation was determined by measuring the loss of [^{3}H]NMS binding sites induced by exposure to 100μM carbachol in JEG-3 cells transiently transfected with mutant mAChR. Previous studies have shown that M2, but not m1 mAChRs, undergo agonist-induced sequestration in JEG-3 cells. G protein coupling efficiency was determined similarly in JEG-3 cells transfected with mutant mAChR and a luciferase reporter gene driven by cAMP response element promoter. We demonstrate that substituting AALS for the VTIL motif in the M2 mAChR abolishes both agonist-induced sequestration and $G_{i\alpha}$ mediated inhibition of cAMP production. In contrast, substituting VTIL for the AALS motif in m1 mAChR confers limited capacity for agonist-induced sequestration and only partially inhibits cAMP production.

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MUTANT M_1 MUSCARINIC RECEPTORS WITH ENHANCED AGONIST ACTIVITY

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A mutant M_5 muscarinic receptor with mutations (S465Y and T466P) at the C-terminus of TM VI displayed constitutive activity and increased agonist potency and binding affinity [Spalding et al. (1995) J. Pharmacol. Exp. Therap. 275: 1274-1279]. These residues are highly conserved in human M_1 receptors. In order to determine if constitutive activity can be induced by equivalent double mutations in M_1 receptors, and which residues are responsible for enhanced activity, we created and characterized mutant M_1 receptors in both A9 L and CHO cells.
The double mutant receptor [M_1(S388Y, T389P)] exhibited enhanced agonist sensitivity and potency when expressed in either A9 L or CHO cells at comparable levels with M_1(WT) receptors. The mutant receptor showed a small degree of constitutive activity at high expression levels. Acetylcholine (ACh) displayed a 50-fold increase in potency at M_1(S388Y, T389P) receptors. Choline, a normal component of growth media, displayed weak partial agonist activity at low expression levels and full agonist activity at high levels of M_1(S388Y, T389P). M_1(S388Y) receptors showed similar agonist activity profiles as M_1(S388Y, T389P) receptors when expressed at a comparable levels in A9 L cells. Choline exhibited full agonist activity yet low potency, while ACh also displayed a 37-fold increase in potency at M_1(S388Y) receptors.
To examine the residues involved in agonist binding and activity at M_1(S388Y, T389P) receptors, we created a third mutation (N382A). ACh displayed low agonist potency at M_1(N382A, S388Y, T389P) and M_1(N382A) receptors, suggesting that N382 is involved in agonist binding and receptor activation in wild-type and mutant M_1 receptors. The mutant receptors thus provide useful tools for exploring the molecular details of receptor activation processes. This work was supported by NS 01493, and NS 35127.

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MUTANT M_1 MUSCARINIC RECEPTORS WITH INCREASED AGONIST AFFINITY AND G PROTEIN COUPLING

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Human M_1(S388Y) and M_1(S388Y, T389P) mutant receptors exhibited enhanced agonist sensitivity and were activated by choline. Here we present the ligand binding properties of M_1(S388Y) and M_1(S388Y, T389P) receptors. Wild type (WT) and mutant M_1 receptors were stably expressed in A9 L cells and membranes were collected for radioligand binding assays. The two mutant receptors exhibited almost the same binding profiles for muscarinic antagonists, including [^{3}H]-(R)-QNB, *l*-hyoscyamine and pirenzepine, and only modest (within 4-fold) decreases in affinity as compared with M_1(WT) receptors. In contrast, the two mutant receptors showed greatly increased binding affinity for muscarinic agonists. Classical full muscarinic agonists, [i.e., acetylcholine (ACh), carbachol, and oxotremorine-M] showed multiple affinity states at both M_1(WT) and the mutant receptors. All tested partial agonists, including choline and methylcarbachol, also exhibited multiple binding sites at M_1(S388Y, T389P), while only arecoline bound to two sites at M_1(S388Y) receptors. High affinity agonist binding was shifted in the presence of 100 μM GppNHp at M_1(S388Y, T389P) receptors, however high affinity ACh binding to M_1(S388Y) was not reduced in the presence of 100 μM GppNHp. Molecular modeling studies suggested that S388Y and T389P mutations change the geometry of key amino acid residues within the M_1 receptor binding pocket. The results indicate that the mutations produce conformational states favorable for agonist binding and G protein coupling. Compounds that bind and stabilize active receptor conformations might be useful in the treatment of neurological disorders such as Alzheimer's disease. This work was supported by NS 01493 and NS 35127.

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TYR381 IN TRANSMEMBRANE DOMAIN SIX OF THE RAT M_1-MUSCARINIC ACETYLCHOLINE RECEPTOR PLAYS A DUAL ROLE IN RECEPTOR FUNCTION.

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An alanine scanning mutagenesis approach has shown that Tyr381, in the rat M_1-muscarinic acetylcholine receptor, plays a major role in both ligand binding and functional response. Tyr381 forming hydrogen-bond interactions with the ester-oxygen atoms of acetylcholine (ACh) was the simplest hypothesis derived from radioligand binding studies using a series of choline-, quinuclidine- and tropine-based ligands (Ward and Hulme, 1997).
To investigate the role of this residue further, the Tyr381Phe mutant was made and characterised. Radioligand binding studies and phosphoinositide turnover experiments showed that ACh binding affinity decreased 33 fold and acetylcholine potency in the functional response was reduced 66 fold, when compared to wild-type. These data were also compared with data from the Tyr381Ala mutant which showed a 28 fold decrease in ACh binding affinity and 2754 fold reduction in ACh potency in the functional response, when compared to wild-type.
The Tyr381 mutant and wild-type receptors were also probed with ACh-reversed ester. The Tyr381Phe mutant had a 7 and 21 fold decrease in binding affinity and potency in the functional response, respectively, when compared to wild-type, whilst Tyr381Ala gave a similar decrease in binding affinity (5 fold) and a larger decrease in potency (195 fold), when compared to wild-type.
These data for ACh and ACh-reversed ester were used to produce efficacy calculations suggesting that the hydroxyl group of Tyr381 is involved in hydrogen bonding to the ester-oxygen atoms of ACh in the receptor's ground state. The benzene ring, of Tyr381, seems to play a role either in binding to a moiety of ACh or in forming an intra-molecular interaction that stabilises the receptor's activated state.

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Ward, S.D.C. and Hulme, E.C. (1997) *Soc. Neurosci. Abstr.* 23, 1750.

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EXPRESSION AND FUNCTIONAL CHARACTERIZATION OF FLUORESCENT CHIMERIC HM1 MUSCARINIC RECEPTORS

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Structural analysis of muscarinic acetylcholine receptors (mAChRs) is hindered by several factors such as their paucity and their subtype multiplicity in natural sources, their molecular heterogeneity due to glycosylation and proteolytic processes and finally the transmembrane location of their ligand binding domain. Several of these problems should be overcome by protein engineering of a well-defined mAChR subtype and introduction of appropriate and non deleterious modifications. Different recombinant human M1 (hM1) mAChRs were designed and stably expressed in HEK 293 cells: 1-Fluorescent chimera have been obtained through fusion of the N-terminus of the receptor with enhanced green fluorescent protein (EGFP); 2-Potential glycosylation sites were suppressed and a large part of the intracellular i3 loop was deleted; 3-An histidine tag sequence was introduced at the C-terminus of the hM1 receptor while a FLAG epitope was either located at the N-terminus of the receptor or inserted into its shortened i3 loop. All these chimeric receptors display high levels of stable expression (up to 900'000 receptor sites per cell). Proper folding and targeting to the plasma membrane was obtained upon addition of a signal peptide to the N-terminal domain of the protein. Ligand binding and functional properties, which are very similar to those of the wt hM1 receptor, indicate that these fluorescently-labelled receptors are valuable tools for further functional, biochemical and structural (site-directed photolabelling) studies. Selection of overexpressing cells through fluorescence measurements and cell sorting, molecular homogeneity of the mAChR as well as facilitated detection and purification of the protein and/or of peptide fragments through differential tagging of the receptor are expected from such a protein tailoring approach.

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DEVELOPMENT OF A DISULFIDE CROSS-LINKING STRATEGY TO STUDY MUSCARINIC RECEPTOR STRUCTURE

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Currently, most of our knowledge about the structure of G protein-coupled receptors (GPCRs) is derived from mutagenesis and molecular modeling studies. To gain more direct insight into GPCR structure, we have developed a disulfide cross-linking strategy using the rat M3 muscarinic receptor as a model system. Initially, the M3 receptor subtype was modified as follows: An HA-epitope tag was attached to the N-terminus, the central portion of the third cytoplasmic loop (i3) was replaced with a factor Xa (fXa) recognition sequence (triple repeat), and most native cysteine residues were replaced with serine or alanine (remaining cysteine residues: C140, C220, and C532). The resulting mutant receptor (referred to as M3'-Xa) showed ligand binding and G protein coupling properties similar to the wild type receptor. Treatment with fXa of membrane lysates prepared from M3'-Xa-transfected COS-7 cells, followed by Western blot analysis using antibodies directed against the N- and C-termini of the receptor, showed that M3'-Xa could be efficiently cleaved by the fXa protease. In the next step, pairs of cysteine residues were reintroduced (one cysteine N-terminal and the other one C-terminal of the fXa recognition sequence) into distinct intracellular regions of M3'-Xa, such as the second intracellular loop (i2) and the N- and C-terminal portions of the i3 loop. Membrane lysates prepared from COS-7 cells transfected with the resulting constructs were treated with fXa, followed by incubation with the oxidizing agent, copper phenanthroline (50 μM). Cross-linked products were identified via immunoprecipitation and Western blot analysis. Similar cross-linking experiments were also carried out with pairs of receptor fragments generated by 'splitting' the M3 receptor in the i3 loop. Preliminary data indicate that a cysteine residue introduced at the beginning of the i2 loop (position I169) can be cross-linked to several sites within the C-terminal segment of the i3 loop. This strategy, which is also applicable to other classes of GPCRs, should eventually lead to a refined structural model of the cytoplasmic surface of the M3 receptor protein.

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DEVELOPMENT OF A SANDWICH ELISA STRATEGY TO STUDY MECHANISMS INVOLVED IN MUSCARINIC RECEPTOR ASSEMBLY

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The muscarinic acetylcholine receptors, like all other G protein-coupled receptors (GPCRs), are predicted to consist of a bundle of seven transmembrane helices (TM I-VII) that are connected by various extracellular and intracellular loops. Currently, little is known about the molecular interactions that are critical for the proper assembly of the transmembrane receptor core. By using the rat M3 muscarinic receptor as a model system, we have previously shown that coexpression of two receptor fragments (referred to as M3-trunk and M3-tail) generated by 'splitting' the M3 receptor subtype in the third intracellular loop results in the reconstitution of functional muscarinic receptors (Schöneberg *et al.*, J. Biol. Chem. *270*, 18000-18006, 1995). We initially demonstrated, by employing a newly developed sandwich ELISA strategy, that the two receptor fragments, M3-trunk and M3-tail, directly associate with each other. In subsequent experiments, this ELISA system was used to identify amino acids that are required for proper receptor assembly. Point mutations were introduced into M3-trunk or M3-tail, and the ability of these mutations to interfere with proper fragment assembly was studied in cotransfected COS-7 cells. Three point mutations were identified (P242A (TM V), P505A (TM VI) and P540A (TM VII)) which, when introduced into M3-trunk (P242A) or M3-tail (P505A and P540A), respectively, strongly reduced fragment association. The mutated proline residues are conserved in most GPCRs of the rhodopsin family, suggesting that they play similar roles in other GPCRs. The approach described here should eventually led to an improved structural model of the transmembrane core of GPCRs.

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MUSCARINIC RECEPTOR-ACTIVATED ARACHIDONIC ACID RELEASE IS ENHANCED BY CYTOKINES IN A2058 HUMAN MELANOMA CELLS

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Neural degeneration of cholinergic neurons such as occurs during Alzheimer's disease, may involve cytokine-mediated inflammatory processes. A 85-kDa phospholipase A2, cPLA2, has been implicated in ischemic neural loss (Bonventre J.V. et al. Nature 390:622) and is elevated in Alzheimer's brains (Stephenson D.T. et al. Neurobiol of Disease 3:51). We investigated possible relationships between cytokine and muscarinic receptor signal transduction mediated by phospholipase A2.

We have previously demonstrated that the human melanoma cell line A2058 expresses only the m5 subtype of muscarinic receptor (Kohn et al. J Biol Chem 271:17476). In A2058 cells, treatment with the cholinergic agonist, carbachol (CC) induces a 2-4 fold increase in arachidonic acid release over basal levels. CC-induced arachidonic acid release is blocked by the muscarinic antagonist, atropine. The CC-induced arachidonic acid release is potentiated 2-3 fold by pretreatment of A2058 cells with either of the inflammatory cytokines, tumor necrosis factor-α (TNFα) or interleukin 1-β (IL-1β). Enhancement of CC-induced arachidonic acid release by TNFα pretreatment peaks near one hour, consistent with a transcriptionally derived event. Western analysis of IkBα, the inhibitory subunit of the NFkB complex, suggests that both cytokines are capable of activating kB-driven transcription. Pretreatment of A2058 cells with either of the proteasome inhibitors, MG-115 or MG-132, block the cytokine-dependent degradation of IkBα but do not affect the enhancement of CC-induced arachidonic acid release. Western analysis demonstrates that both cytokines can trigger the phosphorylation (and activation) of p38 MAP kinase. Furthermore, pretreatment of A2058 cells with the p38 kinase inhibitor, SB202190, ablates cytokine-dependent augmentation without interfering with CC-induced AA release. Finally, no change in the phosphorylation state of the p44/42 MAP kinases follows cytokine treatment. Thus, inflammatory cytokines may regulate muscarinic signaling through the p38 MAP kinase pathway. Additionally, this study provides evidence that potentiation of muscarinic receptor-mediated signal transduction may be a direct result of inflammation.

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MUSCARINIC ACTIVATION OF MAPK IN PC12 CELLS

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Stimulation of muscarinic receptors (mAChR) activates a variety of downstream pathways, several of which lead to the phosphorylation and activation of MAPK. MAPK then plays a crucial role in regulating cell growth and differentiation. However, much of the work examining these pathways has been performed in non-neuronal transfected cells overexpressing the mAChR receptors. Here, we examine the activation of MAPK in a PC12 cell line that endogenously expresses mAChR. By Western blot analysis using an antibody specific for the phosphorylated form of MAPK (pMAPK), we observed a dose-dependent increase in MAPK phosphorylation in cells stimulated with .1 μM to 100 μM carbachol. There was no change in total levels of MAPK protein. The maximal response, a 100% increase in pMAPK levels over baseline as measured by quantitative chemifluorescence technology, occurred after 5 minutes of stimulation with 100 μM carbachol and was reduced to baseline by 30 minutes of continuous stimulation. In the presence of atropine, there was no MAPK phosphorylation in response to carbachol. In fact, atropine treatment reduced levels of pMAPK below those of unstimulated cells, suggesting basal levels of MAPK activation by constitutively released acetylcholine. To investigate the receptors responsible for carbachol activation of MAPK in PC12 cells, we determined the mAChR subtypes present using RT-PCR and immunoprecipitation techniques. Using primers specific for the five mAChR subtypes, we amplified fragments of the appropriate sizes for M_1 and M_4, and confirmed the identity of the bands with restriction digests. Using subtype-specific antibodies, we immunoprecipitated M_1 and M_4. Studies using receptor selective antagonists and inhibitors of intracellular signaling pathways are in progress to identify molecular mechanisms of muscarinic activation of MAPK. Our studies indicate that PC12 cells may provide an excellent system for investigating mechanisms of MAPK activation by endogenous mAChR.

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NERVE GROWTH FACTOR UTILIZES COMMON SIGNALING PATHWAYS TO DIFFERENTIALLY REGULATE M_4 MUSCARINIC AND ADENOSINE A_{2A} RECEPTOR mRNA.

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We have examined the role of various nerve growth factor (NGF)-activated signaling pathways in regulating mRNA expression of two G-protein coupled receptors (GPCRs), the M_4 muscarinic acetylcholine receptor and the adenosine A_{2a} receptor in the rat PC12 cell line. NGF responses are mediated by two receptors, the low affinity p75 receptor and the high affinity TrkA receptor.

We have previously shown NGF-induced stabilization of M_4 mRNA is mitogen activated protein kinase (MAPK)-dependent. Here we show that the NGF-induced increase in M_4 steady state mRNA requires Ras and Src activity as PC12 cells expressing dominant negative Ras or Src proteins fail to show an increase in M_4 mRNA in response to NGF treatment. Futhermore, PC12 cells expressing the mutant TrkA receptor (Y490/785F), which fails to bind SHC and PLC-γ also fail to up-regulate M_4 receptor mRNA in response to NGF.

We next examined potential signaling pathways involved in NGF-induced regulation of A_{2a} mRNA. NGF induced a 56% decrease in steady state A_{2a} mRNA following 3 d treatment. The MEK inhibitor, PD98059 prevented 40% of the NGF-induced decrease in A_{2a} mRNA, indicating a role for MAPK in regulating A_{2a} mRNA. PC12 cells expressing a dominant negative Ras or Src protein did not down-regulate A_{2a} mRNA after NGF treatment. The TrkA tyrosine kinase inhibitor, AG879, inhibited NGF regulation of A_{2a} mRNA whereas NGF was without effect on both the A_{2a} and M_4 receptor mRNA in the TrkA deficient PC12nnr5 cell line. However, NGF-mediated regulation of A_{2a} and M_4 mRNA was rescued in nnr5 cells transfected with wild-type TrkA. These data implicate TrkA, Ras, MAPK and Src activation in NGF regulation of M_4 and A_{2a} receptor mRNA and demonstrate NGF utilizes common signaling mechanisms to differentially regulate GPCR mRNA.

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INVESTIGATION OF THE MULTIPLE AGONIST AFFINITY STATES AT THE M_1 MUSCARINIC ACETYLCHOLINE RECEPTOR IN INTACT AND BROKEN CELL BINDING EXPERIMENTS.

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The presence of multiple agonist affinity states in competition binding studies, conducted on broken cell preparations, is often explained on the basis of agonist distribution between free receptor and receptor-G protein complexes. Most studies utilizing intact cells, however, tend not to reveal multiphasic agonist binding curves, as the excess GTP within the cell does not allow detectable accumulation of the ternary complex.

In the present study, the binding characteristics of the full agonists acetylcholine and carbachol, as well as the partial agonists pilocarpine, McN-A-343, and xanomeline, were determined in competition studies with the antagonist [^{3}H]N-methylscopolamine, in both intact and broken Chinese hamster ovary cells, stably expressing the human M_1 or M_2 muscarinic acetylcholine receptor (mAChR). The data obtained from the M_2 mAChR studies were fully consistent with the predictions of the ternary complex model. In contrast, studies conducted on the M_1 mAChR appeared to deviate from the simple model predictions. In both M_1 homogenates and intact cells, the full agonists retained biphasic binding isotherms that could not be modulated by ions or guanine nucleotides. The high-affinity binding state, however, was dependent on incubation temperature. These findings suggest that the evolution of multiple agonist affinity states in binding experiments may be dependent on different factors, according to the mAChR subtype involved.

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A NOVEL AND SIMPLE APPROACH TO DETECTING PARTIAL AGONIST EFFICACY IN RADIOLIGAND BINDING EXPERIMENTS.

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The ascription of efficacy to muscarinic acetylcholine receptor (mAChR) agonists in radioligand binding experiments has relied on the interpretation of complex agonist binding isotherms and their regulation by ions, guanine nucleotides and other factors. This is particularly evident for full agonists, but not necessarily so for partial agonists. In the present study, an alternative approach is presented. Using homogenate preparations from Chinese hamster ovary cells, stably expressing the human M_1 or M_2 mAChR, the binding properties of the full agonists acetylcholine and carbachol, the partial agonists pilocarpine and McN-A-343, and the antagonist atropine, were investigated in competition experiments against [^{3}H]N-methylscopolamine. The full agonists displayed complex binding curves and were modulated by guanine nucleotides, whereas the binding of the partial agonists and atropine was monophasic and minimally perturbed by the nucleotides. However, when the acetylcholine competition curves were reconstructed in the presence of graded concentrations of either partial agonist, a significant redistribution of the fraction of high-affinity state recognized by acetylcholine was observed. In contrast, atropine had no significant effect on the fraction of either the high or low affinity acetylcholine binding components. These results indicate that changes in the profile of full agonist binding isotherms, when constructed in the presence of a partial agonist, may be more sensitive indicators of partial agonist efficacy than regular assays that directly measure partial agonist binding.

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COMPARISON OF GTP-γ-^{35}S AND ^{3}H-NMS BINDING IN CHO CELLS STABLY EXPRESSING HUMAN M1-M5 MUSCARINIC RECEPTORS USING SCINTILLATION PROXIMITY ASSAYS

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Mammalian cells transfected with human G-protein-coupled receptors are convenient reagents widely used in radioligand binding and functional assays for estimating apparent affinities and efficacies of potential pharmaceutical agents. We have developed scintillation proximity assays (SPA) for GTP-γ-^{35}S binding and ^{3}H-NMS binding in membranes isolated from Chinese hamster ovary cells stably expressing human M1-M5 muscarinic receptors. Data comparing binding profiles in these assays for the muscarinic agonists xanomeline, carbachol, acetylcholine, oxotremorine, oxotremorine-M, arecaidine propargyl ester, pilocarpine, RS86 and muscarine, as well as the muscarinic antagonists atropine, pirenzepine, 4-DAMP and AF DX-116 will be presented. Affinity data from both assays at individual subtypes of muscarinic receptors were strongly correlated. Antagonists displayed expected subtype selectivity;

Inhibition of Oxo-M Stimulated GTP-γ-^{35}S Binding

	K_i, (nM)				
	M1	M2	M3	M4	M5
Atropine	1.5	0.9	0.9	0.6	1.0
Pirenzepine	7	179	384	22	186
4-DAMP	0.6	5.5	1.9	1.8	2.1
AF DX-116	1229	31	3011	184	>10000

however, standard full agonists displayed apparent selectivity for the m2 subtype. GTP-γ-^{35}S and ^{3}H-NMS binding to CHO membranes performed in SPA format provide a means for rapid assessment of ligand affinities across all five subtypes of muscarinic receptors simultaneously.

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EFFECT OF AGONISTS ON [^{35}S]-GTPγS BINDING TO HM1-CHO CELL MEMBRANES

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We compared agonists binding to M_1 receptors in transfected CHO cells with their effect on [^{35}S]-GTPγS in the same buffer. Full agonists discriminated two receptor states with high/low affinity in the absence of guanyl nucleotides, and had a homogeneous low affinity in the presence of GTP or GDP. They induced [^{35}S]-GTPγS binding to high affinity binding sites. The effect of agonist-bound receptors was catalytic (each agonist-bound receptor induced [^{35}S]-GTPγS binding to several G proteins), and the agonists' EC_{50} value increased in the presence of GDP. The low EC_{50} values observed in the absence of GDP was not due to the presence of spare receptors.

These results suggested that muscarinic agonists were capable of activating low affinity "uncoupled" receptors (in the presence of μmolar GDP or GTP concentrations) probably by accelerating the GDP dissociation. Muscarinic agonists also activated "G-protein coupled" (high affinity) receptors which accumulate at very low guanyl nucleotides concentrations (50 pM [^{35}S]-GTPγS) and in the presence of agonists : they probably increased the GTPγS association rate constant.

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A STUDY OF M1 RECEPTOR COUPLING TO INHIBITORY G PROTEINS IN CHO CELLS VIA GTPγS BINDING DETERMINED WITH ANTI-G PROTEIN ANTIBODIES.

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Muscarinic M1 receptors stimulate phospholipase Cβ by coupling to members of the Gq family of GTP binding proteins. Recent literature reports have shown partial blockade by pertussis toxin (PTX) of GTPγS binding resulting from agonist-stimulation of muscarinic M1 and M3 receptors in transfected cell lines, suggesting that these receptors couple to Gi in addition to Gq proteins. This phenomenon was investigated using membranes from CHO M1 cells and a novel binding assay to determine GTPγS binding to specific classes of Gα subunits. GTPγ^{35}S binding was determined via scintillation proximity assay using wheatgerm agglutinin-coated beads for whole membranes or anti-rabbit IgG-coated beads in conjunction with anti-Gαq/11 or anti-Gαi antibodies. Using membranes from monolayer cultures, PTX inhibited oxotremorine M-stimulated GTPγS binding to whole membranes by 70%, but had no effect on agonist-stimulated binding to Gαq/11. Direct coupling of the M1 receptor to Gαi proteins was demonstrated via GTPγS binding measured with anti-Gαi(1-3) using monolayer membranes (M), but was not significant when determined using membranes from suspension-adapted cells (S). For binding to Gαq/11, M membranes showed a higher potency for the full agonist oxotremorine M and higher efficacy for the partial agonist pilocarpine than S membranes, indicating that receptor reserve was greater for M than S. Thus the degree of receptor reserve for coupling of M1 to Gαq/11 appears to determine whether coupling to Gαi occurs.

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AN ANTI-G PROTEIN SCINTILLATION PROXIMITY ASSAY FOR M1-M5 RECEPTOR-MEDIATED GTPγS BINDING.

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Receptor-dependent GTPγS binding has traditionally been determined using whole membrane preparations and rapid filtration to separate bound from free ligand. Recent literature reports have demonstrated GTPγS binding using specific antibodies to Gα subunits captured by immunoprecipitation, and this method has been shown to improve signal to noise for M1 receptors (Reever et. al., Life Sci 56: 1032, 1995). We have extended this approach by developing a GTPγS binding assay using anti-Gα primary antibodies coupled with Scintillation Proximity Assay (SPA) beads coated with second antibodies. GTP-γ-^{35}S binding to whole membranes was performed using wheatgerm agglutinin-coated SPA beads. In the anti-Gα assay, ^{35}S-labeled membranes were solubilized with 0.27 % NP40 followed by addition of rabbit anti-Gαq11 for M1, M3, and M5 receptors, or rabbit anti-Gαi(1-3) for M2 and M4 receptors. Antibody-G protein complexes were captured with anti-rabbit IgG-coated SPA beads. Although the antibody based assay did not significantly improve signal to noise ratios for M2 and M4 receptors, a 12-17 fold increase in agonist-stimulation (expressed as percent increase over basal) was achieved for the M1, M3, and M5 receptors. M1 receptors were found to couple to both Gαq/11 as well as Gαi proteins, while M2 receptors coupled only to the Gαi family. The antibody capture assay in SPA format improves signal to noise for receptors linked to phospholipase Cβ , is readily automatable, and is a useful tool for exploring promiscuous coupling of receptors in transfected cells.

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CLOZAPINE BEHAVES AS A PARTIAL AGONIST AT DIFFERENT MUSCARINIC RECEPTOR SUBTYPES EXPRESSED IN CHO CELLS.

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The atypical antipsychotic drug clozapine has been reported to be a full and selective agonist at the cloned human M_4 receptor and an antagonist at the other muscarinic receptor subtypes (Zorn et al. Eur. J. Pharmacol.-Mol.Pharm.Sect. 269, R1-R2, 1994). However, we have found that at the M_4 receptor clozapine behaves as a partial agonist, rather than as a full agonist, with intrinsic activity changing as a function of the receptor density and coupling efficiency of the receptor to effector molecules (Olianas et al., Br. J. Pharmacol. 122, 401-408, 1997). In the present study, we have investigated whether the drug could elicit similar effects at other muscarinic receptor subtypes. In CHO cells expressing either the M_1 or the M_3 receptor subtype, clozapine elicited a concentration-dependent stimulation of [^{3}H]-inositol phosphates accumulation with EC_{50} values of 40 and 170 nM, respectively. In CHO cells expressing the M_2 receptor, clozapine inhibited forskolin-stimulated cyclic AMP accumulation and enhanced [^{35}S]GTPγS binding to membrane G proteins with EC_{50} values of 300 and 150 nM, respectively. The agonist effects of clozapine were completely antagonized by atropine. At each receptor subtype, the intrinsic activity of clozapine was lower than that of the full agonist carbachol and, when the compounds were combined, clozapine potently reduced the receptor responses to carbachol. These data indicate that clozapine behaves as a partial agonist at different muscarinic receptor subtypes and may provide new hints for understanding the receptor mechanisms underlying the antipsychotic efficacy of the drug.

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M_1 MUSCARINIC RECEPTORS POTENTIATE CRH-STIMULATED ADENYLYL CYCLASE ACTIVITY IN MEMBRANES OF RAT FRONTAL CORTEX.

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Stimulation of cortical M_1 muscarinic receptors has been identified as a preferential target for drugs effective in the treatment of cognitive deficits. Moreover, it has been proposed that potentiation of central corticotropin releasing hormone (CRH) transmission may be beneficial in the treatment of Alzheimer's disease (Behan et al., Nature, 378, 284-287, 1995). Here we report that in membranes of rat frontal cortex acetylcholine (ACh) and other cholinergic agonists potentiate the stimulation of adenylyl cyclase activity elicited by CRH. Oxotremorine-M, carbachol and methacoline were as effective as ACh, whereas oxotremorine and arecoline were much less effective. The facilitating effect of ACh was potently blocked by the M_1 antagonists R-trihexyphenidyl, telenzepine and pirenzepine, whereas the M_2-M_4 antagonists himbacine, methoctramine, AQ-RA 741 and AF-DX 116 were less potent. The M_1 potentiation was pertussis toxin-insensitive and not affected by the phospholipase C inhibitor U-73122, the protein kinase inhibitor staurosporine and by arachidonic acid metabolism inhibitors. Conversely, the M_1 potentiation was reduced by the G_q antagonist GPant-2A and by membrane treatment with the GDP-bound form of the α subunit of transducin. Addition of exogenous G protein $\beta\gamma$ subunits mimicked the muscarinic potentiation. The data indicate that M_1 and CRH receptors are colocalized on cellular structures of rat frontal cortex and that M_1 stimulation positively regulates CRH receptor activity through $\beta\gamma$ subunits of G_q.

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ACTIVITY OF SELECTIVE MUSCARINIC AGONISTS AT HUMAN M_1 RECEPTORS EXPRESSED AT VARYING LEVELS IN A9 L CELLS

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Selective M_1 muscarinic agonists might be useful in the treatment of Alzheimer's disease. Numerous compounds have been synthesized and characterized for muscarinic agonist activity, and a few have entered into clinical studies. Relatively few studies have compared the activity of these compounds at M_1 receptors under the same conditions. Here we compare the activity of three M_1 agonists at human M_1 receptors expressed in A9 L cells. 3-(3-Hexyloxy-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (xanomeline), (4R,S)-Z-1-azabicyclo[2.2.1]heptan-3-one-O-[3-(3'-methoxyphenyl)-2-propynyl]oxime [(±)-PD151832] and CDD-0102 were synthesized according to published procedures. M_1 receptors were stably expressed in A9 L cells at low and high levels. Agonist activity was assessed by examining [^{3}H]-inositol phosphate (IP) production. Each agonist displayed robust activity at M_1 receptors expressed at high levels. Xanomeline was the most potent (EC_{50} = 9.4 nM) and efficacious [59 % of carbachol (CCh)], followed by CDD-0102 (3.8 μM; 53 % of CCh) and (±)-PD151832 (6.6 μM; 42 % of CCh). At lower expression levels, xanomeline also was the most potent (24 nM; 41 % of CCh), followed by CDD-0102 (4.8 μM; 42 % of CCh) and (±)-PD151832 (5.0 μM; 58 % of CCh). In each assay, IP levels dropped dramatically at higher concentrations of xanomeline and (±)-PD151832. In contrast to the stimulation of IP levels, this effect of xanomeline was not blocked by *l*-hyoscyamine. The data indicated that xanomeline is the most potent of the ligands tested, yet its potency and activity depended upon the level of receptor expression. Receptor expression level is an important determinant of agonist activity. The work was supported by NS 01493, NS 31173 and NS 35127.

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MUSCARINIC RECEPTOR SUBTYPES AND AGONIST-MEDIATED SIGNAL TRANSDUCTION IN CULTURED CELL LINES

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There are at least five subtypes (M1-M5) or more (M6, Mi, Ms) of muscarinic receptors (1-3). Two signal transduction mechanisms, attenuation of hormone-stimulated c-AMP formation and phosphatidylinositol (PI) hydrolysis, are coupled to M receptors in NG-108-15 neuroblastoma x glioma (NC) and 131-1N1 human astrocytoma (AC) cells, respectively. Using selective antagonists, the receptors in NC and AC cells were characterized as M2 and M3 sub-types (1,2). In a search to find selective agonists, we investigated the effects of several muscarinic agonists on these transduction mechanisms (3). The potencies of full agonists for c-AMP attenuation has the order: carbachol > 5-methylfurmethide (5MFT) > dl-muscarine. All are full agonists and produced maximum inhibition of 73-76%. These three agonists maximally stimulated PI hydrolysis (100%) in AC cells. Their relative potencies have a different order 5-MFT > carbachol > dl-muscarine. Agonists which are considered to be selective for M1 (MCN A345) Ms (5-methoxyfurmethide) and Mi (5-hydroxymethylfurmethide) exhibited low activity on cAMP attenuation in NC cells (maximum inhibition, < 42-46%), and PI hydrolysis in AC cells (maximum stimulation 0-22%). These observations suggest that (a) non-selective full agonists mediate both effects, and (b) selective agonists at M1, Ms and Mi receptors do not mediate these effects. Selective M2 and M3 agonists have yet to be discovered to support the previous observations with antagonists that M2 receptors mediate cAMP attenuation while M3 receptors mediate PI hydrolysis. (Supported by the Smokeless Tobacco Research Council, Inc.).

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EFFECT OF MUSCARINIC STIMULATION ON β-AMYLOID PRECURSOR PROTEIN PROCESSING IN RAT BRAIN AND PRIMARY CULTURES

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Metabolism of β-amyloid precursor protein (APP) can lead to the formation of amyloid β-protein (Aβ), neurotoxic agent found in brains of Alzheimer's patients. An inverse relationship is reported between the levels of Aβ and secreted APP (APPs). Activation of M_1 and M_3 muscarinic acetylcholine receptors (mAChR) increases APPs secretion in cell lines expressing these mAChR and in cerebrocortical slices (Pittel et al., 1996). We examined the effect of muscarinic stimulation on APPs secretion in primary neuronal cultures and in rat brain in vivo. Primary cell cultures derived from the hippocampus and the cortex of 18 days old rat fetuses were used. Exposure of neuronal cultures for 1 h to the non selective muscarinic agonist, carbachol and to the functionally M_1 selective muscarinic agonists, AF102B and AF150(S) (each at 100 μM) significantly increased APPs secretion by 2-6 fold over basal level. These effects were blocked by 10 μM pirenzepine, an M_1 selective antagonist, suggesting the enhancing role of M_1 mAChR in APPs secretion. Studies in vivo indicate that more than one subtype of mAChR might be responsible for APP processing. Administrations of oxotremorine (1 mg/kg; ip) and the cholinesterase inhibitor, physostigmine (0.25 mg/kg; im) to rats reduced the levels of APPs in the soluble fraction of the hippocampi 1 h after the injections by 52 and 44%, respectively, compared to control rats. It seems that following muscarinic stimulation different mAChR subtypes may dominate the regulation of APP processing in the brain (e.g. M_2 or M_4 inhibitory mAChR) although other factors can be considered. The relative interplay between two or more mAChR subtypes (excitatory vs inhibitory) on APPs processing needs further investigation.

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INVESTIGATING THE MECHANISMS OF UPREGULATION OF NEURONAL NITRIC OXIDE SYNTHASE (nNOS) BY THE MUSCARINIC M_1 RECEPTOR

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Recently we have reported that prolonged stimulation of the muscarinic M_1 receptor resulted in upregulation of nNOS protein levels in both N1E-115 mouse neuroblastoma and Chinese hamster ovary (CHO) cells. The former cells express the receptor and enzyme endogenously and the latter express them in a recombinant fashion. In this study we employed a series of pharmacological agents that modulate various second messengers known to be coupled to the muscarinic M_1 receptor and determined their effects on nNOS expression using Western blot analysis. In both CHO and N1E-115 cells, treatment with the muscarinic agonist carbachol (CCh), the Ca^{2+} ionophore, ionomycin or the protein kinase C activator, phorbol 12, 13 dibutyrate, each individually resulted in increased expression of nNOS in comparison to control untreated cells. CCh combined with the membrane-permeant Ca^{2+} chelator, BAPTA/AM, showed no significant effect on enzyme expression levels compared to control. Interestingly, CCh treatment with the protein kinase C selective inhibitor, GF109203X, resulted in the greatest degree of increased nNOS expression, whereas treatment with the protein kinase C inhibitor alone resulted in a modest increase of nNOS expression over control. In contrast, the general NOS inhibitor L-nitroarginine in combination with CCh resulted in suppression of nNOS upregulation. Whereas L-nitroarginine alone reduced nNOS expression in N1E-115 cells, there was no significant effect in CHO cells. Treatment with 500 μM of the NO donor, S-nitroso-N-acetylpenecillamine, resulted in a marked reduction of nNOS expression in both CHO cells as well as in N1E-115 cells. The results of pharmacological intervention indicate that nNOS expression is modulated by multiple second messenger pathways stimulated by the muscarinic M_1 receptor.

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M_2 AND M_4 MUSCARINIC RECEPTOR SUBTYPES COUPLE TO ACTIVATION OF ENDOTHELIAL NITRIC OXIDE SYNTHASE

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Different subtypes of muscarinic cholinergic receptors have been linked to the production of nitric oxide. It has been demonstrated that nitric oxide synthase (NOS) is regulated by Ca^{2+}/calmodulin. In the presence of Ca^{2+}/calmodulin, NOS oxidizes L-arginine to form nitric oxide and L-citrulline. Muscarinic receptor subtypes M_1, M_3 and M_5 couple to activation of phosphoinositide hydrolysis, which in turn can lead to the release of Ca^{2+} from the endoplasmic reticulum. The released Ca^{2+} can then couple with calmodulin to activate NOS. Receptor subtypes M_2 and M_4, however, preferentially couple to inhibition of adenylyl cyclase. It is unclear how the latter receptors can activate NOS. In the present study we demonstrate the M_2 and M_4 receptors can activate endothelial NOS (eNOS), an isoform that is expressed in neurons, where it plays an important role in memory and learning. Chinese hamster ovary (CHO) cells that express M_2 or M_4 muscarinic receptors as well as eNOS were utilized in the present study. Intact cells were stimulated with various concentrations of carbachol. NOS activity was determined by stimulating the cells in the presence of rat fetal lung fibroblast (RFL-6) cells, which generate cyclic guanosine 3',5'-monophosphate (cGMP) in the presence of nitric oxide. We found that both M_2 and M_4 receptors were able to activate eNOS in a concentration-dependent manner. We were further able to show that both M_2 and M_4 receptors caused an increase in the levels of intracellular free Ca^{2+} in a concentration-dependent manner. Chelating extracellular Ca^{2+} with EGTA did not change the Ca^{2+} or NOS activity response, suggesting that intracellular stores of Ca^{2+} are responsible for the NOS activity. However, we were not able to detect increased phosphoinositide hydrolysis upon activation of either M_2 or M_4 receptors.

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MUSCARINIC RECEPTOR-MEDIATED REORGANIZATION OF THE ACTIN CTYOSKELETON IS COUPLED THROUGH THE $G_i\alpha$ 2 PROTEIN IN HUMAN AIRWAY SMOOTH MUSCLE CELLS.

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Muscarinic receptor signaling pathways that couple to contraction of airway smooth muscle (ASM) are also associated with reorganization of the actin cytoskeleton. We have recently shown that a pertussis-sensitive G protein, the small G protein, rho, and a tyrosine kinase are intermediate proteins linking the activation of muscarinic receptors to reorganization of the actin cytoskeleton. The current study was designed to determine which heterotrimeric G protein couples muscarinic receptors to the reorganization of the actin cytoskeleton (measured by an increase in the F/G actin ratio) in ASM. Cultured human airway smooth muscle cells expressing the M_2 muscarinic receptor were grown to confluence. Cells were exposed for 6 days to 10 uM antisense oligonucleotides designed to specifically bind to the mRNA encoding $G_i\alpha 2$, $G_i\alpha 3$, or $G_q\alpha$. A randomly scrambled oligonucleotide served as a control. F/G actin ratios were measured following 5 min of carbachol exposure which we have previously shown to increase the F/G actin ratio indicative of actin reorganization. Cells in parallel wells were harvested for immunoblot analysis of G protein α subunit expression. Results of immunoblots showed that 6 days of oligonucleotide antisense treatment decreased protein expression of the respective G protein α subunit. Antisense depletion of the $G_i\alpha 2$ protein, but not the $G_i\alpha 3$, nor $G_q\alpha$ protein blocked the carbachol-induced increase in the F/G actin ratio. These results show that the $G_i\alpha 2$ protein couples muscarinic receptors to the reorganization of the actin cytoskeleton in airway smooth muscle.

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REGULATION OF MUSCARINIC CHOLINERGIC RECEPTOR ENDOCYTOSIS BY PHOSPHATIDYLINOSITOL 4-KINASE β

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A role for phosphoinositides in the endocytosis of muscarinic cholinergic receptors (mAChRs) has been investigated. Pretreatment of SH-SY5Y neuroblastoma cells with micromolar concentrations of either wortmannin (WT), LY-294002 or phenylarsine oxide (PAO), three chemically distinct agents known to inhibit phosphatidylinositol 4-kinase (PI4K), resulted in both an inhibition of agonist-induced endocytosis of mAChRs and a selective reduction in the ^{32}P-labeling of phosphatidylinositol 4-phosphate. PAO-mediated inhibition of both receptor endocytosis and phosphoinositide synthesis could be fully reversed by inclusion of the bifunctional thiol, 2,3-dimercaptopropanol. Although each of these inhibitors attenuate receptor-mediated second messenger production, mAChR internalization was readily observed under conditions in which PLC activity is essentially abolished ($[Ca^{2+}]<10nM$) and WT inhibited receptor internalization under these conditions by > 75%. Determination of PI4K activity in subcellular fractions of SH-SY5Y cells indicated that WT, LY-294002 and PAO preferentially inhibited enzyme activity in endocytic and cytosolic fractions, a profile consistent with the subcellular distribution of the 110 kDa β-isoform of PI4K, as determined by western blot analysis. Activity of PI4Kβ present in immunoprecipitated cell lysates was inhibited >75% by inclusion of each of the three inhibitors. These results indicate that the ongoing synthesis of phosphoinositides is necessary for mAChR endocytosis and support a role for the PI4Kβ isoform in regulating the phosphoinositide pool required for the maintenance of receptor endocytosis. (Supported by NS 23831, MH 46252, and GM 07767.)

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ARRESTIN-INDEPENDENT INTERNALIZATION OF MUSCARINIC CHOLINERGIC RECEPTORS

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In order to understand what processes contribute to the agonist- induced internalization of subtypes of muscarinic acetylcholine receptor, we analyzed the role of arrestins. While the m2 mAChR has been shown to undergo augmented internalization when arrestins 2 or 3 are over-expressed, the agonist-induced internalization of m1, m3, and m4 mAChRs was unchanged when arrestins 2 or 3 were over-expressed in transiently transfected HEK-tsA201 cells. Furthermore, when a dominant negative arrestin was used to interrupt endogenous arrestin function, there was no change in the internalization of the m1, m3, and m4 mAChR while the internalization of the β_2 adrenergic receptor was completely blocked. Wild-type and GTPase deficient dominant-negative dynamin were used to determine which endocytototic machinery played a role in the endocytosis of the subtypes of mAChRs. Interestingly, when dynamin function was disrupted by over-expression of the GTPase deficient dynamin, agonist-induced internalization of the m1, m3, and m4 mAChR was suppressed. These results suggested that the internalization of the m1, m3, and m4 mAChRs occurs via an arrestin-independent but dynamin-dependent pathway. In order to ascertain whether domains of the arrestin-sensitive m2 mAChR could confer arrestin-sensitivity to arrestin-insensitive subtypes of the mAChRs, chimeric m2/m3 receptors were analyzed for their properties of agonist-induced internalization. The results presented indicated that the third interacellular loop of the m2 mAChR conferred arrestin-sensitivity to the arrestin-insensitive m3 mAChR while that analogous domain of the m3 mAChR conferred arrestin-resistance to the previously arrestin-sensitive m2 mAChR.

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REGULATION OF G-PROTEIN-COUPLED INWARD RECTIFYING K^+ CHANNEL EXPRESSION BY MUSCARINIC RECEPTOR ACTIVATION.

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Stimulation of muscarinic acetylcholine receptors in the heart results in a decrease in the rate of contraction in part through the activation of a G-protein coupled inward rectifying K^+ (GIRK) channel. We used solution hybridization and immunoblot analyses to investigate the effects of muscarinic receptor stimulation on expression levels of the GIRK subunits. Treatment of chick embryos in ovo with muscarinic agonists causes a decrease in mRNA levels encoding GIRK1 and GIRK4 in atria but does not alter GIRK1 expression in ventricles. GIRK1 protein levels also decrease in atria upon muscarinic acetylcholine receptor stimulation. *In ovo* treatment of embryonic chicks with the partial muscarinic agonists pilocarpine and oxotremorine which inhibit adenylyl cyclase but do not stimulate phospholipase C (PLC) in chick heart, also cause decreases in GIRK1 expression. Carbachol treatment of freshly dissociated atrial cells resulted in decreased GIRK1 expression. The regulation of GIRK1 expression by receptor stimulation is lost with continued culture of the atrial cells. The regulation of GIRK expression represents a novel mechanism for the regulation of G-protein coupled effector levels in response to long term receptor activation.

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M_1 MACHR SIGNALING IN CARBACHOL INDUCED POSITIVE INOTROPY IN CULTURED NEONATAL RAT VENTRICULAR MYOCYTES

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In the mammalian heart, vagal innervation is classically associated with m_2 mAChR mediated negative chronotropy and inotropy. However, under conditions in which the m_2 mAChR pathway is inhibited, muscarinic agonists have been shown to produce stimulatory effects on the rate and contractility of the heartbeat. In this study, we investigate the role of the m_1 mAChR subtype and its signaling mechanisms in producing the positive inotropic effects. Contractility measurements performed on intact neonatal rat papillary muscle demonstrate that 300 μM of the non-selective muscarinic agonist carbachol (CCh) produced a 32 ± 4% increase in force of contraction (n=7). In cultured neonatal rat ventricular myocytes (cnrvms) pretreated with pertussis toxin (PTX, 100-200 ng/ml for 18-24 hr), 300 μM CCh produces a 68 ± 23 % increase in diastolic and a 44 ± 15% increase in systolic Ca^{2+} levels, respectively (n=9). This effect is attenuated by the m_1 selective antagonist, pirenzepine (PZP-20 nM) and unaffected by the m_2 selective antagonist, methoctramine (MTC-20 nM), providing evidence for the involvement of the m_1 mAChR receptor subtype in this response. This effect is also attenuated by pretreatment of cells with 50 μM genistein, a protein tyrosine kinase inhibitor, whereas daidzein, the inactive analog of genistein had no effect. Using PI turnover as a measure of phospholipase C (PLC) activation, 300 μM CCh induces a threefold stimulation of PLC activity in cnrvms (n=4). This effect was also attenuated by genistein pretreatment of cells. These data are in agreement with the current knowledge that m_1, m_3, and m_5 mAChRs are coupled via $G_{q/11}$ to PLC in other cell systems. To further substantiate the existence of the m_1 mAChR subtype in cnrvms, we employed single cell RT-PCR and demonstrated the expression of m_1 and m_2 mRNA in these cells. Immunofluorescence studies employing antibodies selective for either m_1 or m_2 subtype of mAChR also revealed the presence of intact m_1 mAChR protein on the surface of cnrvms. We conclude that the activation of m_1 mAChR leads to the stimulation of PLC and tyrosine kinase which then causes the increase of Ca^{2+} transients and subsequent increase in the force of contraction.

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MODULATION OF K^+ CURRENTS BY MUSCARINIC ACETYLCHOLINE RECEPTOR AGONISTS IN CANINE ATRIAL MYOCYTES

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Muscarinic acetylcholine receptors (mAChRs) play an important role in mediating a variety of cellular responses and many of their functions are fulfilled by acting on ion channels. To have better understanding of the interactions between mAChR agonists and ion channels, we assessed the effects of several mAChR agonists on various K^+ currents using whole-cell patch-clamp techniques in isolated canine atrial myocytes. (1) Transient outward K^+ current (I_{to}): ACh (1 μM) caused two-fold increase in I_{to} density at +50 mV. This increase was mediated by stimulation of mAChRs because 1 μM atropine abolished the effects. TMA (0.5 mM), a partial agonist of mAChRs, did not alter I_{to}. 4AP (1 mM), a K^+ channel blocker with properties of M_4 agonism, produced marked reduction of the current density, an effect not antagonized by atropine. (2) Sustained outward current (I_{sus}): No appreciable effects of ACh on I_{sus} were observed. TMA increased the current density by two-fold, and the effects were reversed by atropine. 4AP substantially suppressed I_{sus}, even in the presence of atropine. (3) Delayed rectifier K^+ currents (I_K). ACh completely blocked I_K and the effects persisted after application of atropine. In contrast, TMA and 4AP did not alter I_K, instead induced novel K^+ currents which were highly sensitive to atropine, indicating the role of mAChRs in the current activation. The results indicate that mAChR agonists modulate multiple K^+ currents, either increase or decrease and either via stimulation of mAChRs or by direct interaction with the channels, depending on different agonists and on the target currents.

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M_1 mAChR DOES NOT REGULATE L-TYPE CALCIUM CURRENT WHEN CARBACHOL INCREASES VENTRICULAR MYOCYTE CONTRACTIONS

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Activation of M_1 muscarinic receptors (mAChR) is reported to increase L-type Ca^{2+} current ($I_{Ca(L)}$) in guinea pig ventricular myocytes and increase intracellular Ca^{2+} transients in mammalian ventricular myocytes (Gallo, et al., J. Physiol. 471:41, 1993; Sharma, et al., Circ. Res. 79:86, 1996). We tested this hypothesis in a single ventricular myocytes by measuring $I_{Ca(L)}$ under voltage clamp conditions and contractions in externally-stimulated cells. Basal $I_{Ca(L)}$, measured under conditions with Cs^+ present in pipette (120mM) and bath (10mM) solutions, was unaffected by 100 mM McN-A-343 (M_1-selective), oxotremorine (OXO, M_2-selective) or carbachol (CCh, non-selective). When $I_{Ca(L)}$ was increased by 3-10 nM isoproterenol (ISO), CCh and OXO inhibited by 87±6.7% (n=8) and 50±7.5% (n=4), respectively. Unlike CCh and OXO, McN-A-343 had no atropine-sensitive inhibitory effect on ISO-stimulated $I_{Ca(L)}$. When $I_{Ca(L)}$ was increased by intrapipette cyclic AMP, none of the muscarinic agonists had any effect. Contractions of single ventricular myocytes were increased in 100 mM CCh by 19±6% (from 4.1±0.72 to 5.0±0.84 mm) at a stimulus frequency of 0.2 Hz (n=19) and by 25±10.8% (from 2.8±0.32 to 3.5±0.50 mm) at 1 Hz (n=10). McN-A-343 had no significant effect at either 0.2 Hz (from 3.6±0.51 to 3.5±0.53 mm, n=26) or at 1 Hz (from 3.5±0.36 to 3.4±0.34 mm, n=17). We conclude that: 1) there is a negligible role of M_1 mAChR in either positive or negative regulation of $I_{Ca(L)}$ or contractions, 2) M_2 mAChR are implicated in anti-adrenergic action and 3) maximal elevation of intracellular cyclic AMP suppressed muscarinic inhibition of $I_{Ca(L)}$.

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MULTIPHASIC CALCIUM CURRENTS STIMULATED BY THE HM1 RECEPTORS

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We developed a rapid and sensitive assay of intracellular calcium ions in human embryonic kidney (HEK293) and Chinese hamster ovary (CHO) cells from 96-well plates using a fluorometer equipped with on-line injectors. Compared to the conventional single point measurement with UV-excitable dyes such as Fura-2, this assay was developed in high throughput format with visible light-excitable fluorescent Ca^{2+} dyes Calcium Green-1 and Oregon Green 488 BAPTA-1. Both dyes produce a robust and stable signal upon stimulation of intracellular Ca^{2+} in HEK293 cells. In CHO cells, the fluorescence signal is stable only with Oregon Green 488 BAPTA-1 at room temperature in the presence of an organic anion transporter inhibitor. Changes of cytoplasmic calcium concentrations occur either through release from intracellular calcium stores or by the opening of channels in the membrane. To dissect the possible existence of distinct calcium pools and regulation of calcium influx, we measured calcium responses in endogenous and stably transfected hm1 receptors, in comparison with stably transfected mu-opioid receptors, which also elicit multiphasic Ca^{2+} currents by stimulating pertussis toxin-sensitive G proteins. Our results suggest that mu-opioid and hm1 receptors may activate different intracellular Ca^{2+} stores. Further, we observed at least two calcium influx phases upon agonist stimulation through unidentified Ca^{2+} channels, suggesting the presence of both receptor-operated and capacitative calcium entry.

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ESTIMATION OF ANTAGONIST AFFINITY

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A discussion about the use of Cheng-Prusoff's equation in functional studies flared up in 1993 [1,2,3]. Since then, the subject has not been touched upon. It was concluded that in functional studies of antagonist a functional equivalent of the Cheng-Prusoff equation, fe-C-P, should be used.

Here I question the conclusions reached by the participants in the 1993-discussion.

1) Craig argued that in functional studies one should use a modified form of the Cheng-Prusoff equation, in opposition to antagonism in enzymatic reactions and in displacement binding studies with radio-ligands, of the form

$$K_s = \frac{IC_{50}}{1 + S/EC_{50}}$$

in which K_{is} is the antagonist equilibrium dissociation constant at the activating site in competitive kinetics, IC_{50} equals the antagonist concentration that reduces any agonist-induced response in the absence of inhibitors by half, S is a fixed agonist concentration, and EC_{50} is the apparent agonist equilibrium dissociation constant. In Cheng-Prusoff's equation the true, agonist equilibrium dissociation constant, K_{ss}, replaces EC_{50}. The above equation is referd to as the functional equivalent of C-P's equation, fe-C-P. This equation was derived two years earlier in 1991 by McKinney et al. [4] One problem with the use of C-P in functional studies is the possible presence of a receptor reserve. The functional equivalency form of C-P's equation specifically deals with this problem as demonstrated by Craig.[1]

2) Leff and Dougall likewise warned against the use of C-P in functional studies. These authors further developed the concept of functional equivalency in determining the antagonist equilibrium dissociation constant, K_{is}, when reactions are moreover complicated by not following simple competitive kinetics. Accordingly, they introduced the classic Hill coefficient in an empirical formulation, in order to handle the deviation, a Leff-Dougall expansion = Hill-fe-C-P.[2]

3) Lazareno and Birdsall summarized the subject of determining the K_{is} for antagonist by a comparison of the C-P equation, its functional equivalent, the Gaddum equation, and Schild's analysis. These authors further discussed the use of functional inhibition curves.[3]

Conclusion. My point is that equations (C-P, fe-C-P, Hill-fe-CP, Gaddum, Schild) used to determine the true antagonist equilibrium dissociation constant, K_{is}, are all based on assumptions. When the assumptions are not fulfilled, e.g.; in non-competitive kinetics, none of these equations give a reliable result; and Schild slopes of unity is no guarantee.

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PHARMACOLOGICAL DETERMINATION OF THE INTERACTION OF XANOMELINE WITH M_1 MUSCARINIC ACETYLCHOLINE RECEPTORS.

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Previous finding from our laboratory suggest that the M_1 muscarinic acetylcholine receptor (mAChR) agonist xanomeline exhibits a novel mode of interaction that involves persistent and, possibly, non-competitive binding. The present study used both functional and binding assays to examine this interaction in greater detail. Chinese hamster ovary cells transfected with the genes for the M_1 mAChR and neuronal nitric oxide synthase (nNOS) were used. Functional assays measured either agonist-mediated nNOS activity, via the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline, or changes in intracellular calcium concentrations. In both assays, the presence of increasing concentrations of the mAChR antagonists atropine or pirenzepine yielded parallel dextral shifts of the concentration-response curves to carbachol and xanomeline. The pK_B values of atropine were not significantly different between the agonists in either assay, suggesting a competitive interaction. A similar finding was observed with pirenzepine in the calcium assay, but not in the citrulline assay. Pretreatment of cells with xanomeline, followed by extensive washout, resulted in elevated basal levels of nNOS activity that were suppressed by atropine or pirenzepine in a concentration-dependent manner. Assuming a competitive interaction, the pK_B values were in good agreement with those estimated from the functional assays. Radioligand binding studies showed that the presence or absence of various concentrations of xanomeline had no significant effect on the potency of atropine or pirenzepine in inhibiting the binding of [^{3}H]N-methylscopolamine. This study indicates that xanomeline interacts with the classical binding site on M_1 mAChRs in a competitive manner.

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DETERMINATION OF THE INTRINSIC FUNCTIONAL MUSCARINIC ACTIVITY OF XANOMELINE

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Xanomeline, a selective muscarinic agonist recently evaluated in clinical trials, reduced psychotic-like behaviors and improved cognition in Alzheimer's disease patients. In order to provide a valid comparison for the functional muscarinic receptor subtype selectivity of xanomeline, the degree of receptor reserve inherent in cell lines transfected with muscarinic receptors was determined. Receptor reserve ratios (K_d/EC_{50}) for M_1- M_5 cell lines were calculated to be 27, 4, >1000, 3, and >1000, respectively. Partial alkylation studies revealed xanomeline had virtually no intrinsic efficacy or potency as an agonist at M_3 and M_5 receptors. Relative to a full agonist, the order of intrinsic efficacy for xanomeline was shown to be $M_4 > M_2 > M_1$ (71, 40, and 7%). Xanomeline's intrinsic potency was greatest at M_4 (6 nM), moderate at M_1 (68 nM), and weakest at M_2 (475 nM). Thus, xanomeline was found to be functionally most active at the M_4 receptor subtype and less active at the M_1 and M_2 receptor subtypes with respect to both efficacy and potency. The clinical efficacy of xanomeline may be attributable to its M_1/M_4 agonist activity.

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MUSCARINIC RECEPTOR CHARACTERIZATION AND NEUROCHEMICAL EFFECTS OF THE ANTIPSYCHOTIC-LIKE MUSCARINIC AGONIST PTAC.

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In recent clinical trials with Alzheimer's disease patients, the muscarinic agonist xanomeline decreased psychotic-like behaviors including delusions and hallucinations, suggesting muscarinic agonists may have antipsychotic activity. Therefore, we have investigated the effects of a series of muscarinic agonists in models predictive of antipsychotic activity and report the effects of (5R,6R) 6-(3-propylthio-1,2,5-thiadiazol-4-yl)-1-azabicyclo[3.2.1] octane (PTAC). In radioreceptor binding assays, PTAC had high affinity (Ki values < 1 nM) for the 5 muscarinic receptor subtypes, but was not selective for any subtype. However, PTAC had at least 87 fold less affinity for dopamine (DA) D1-D5, sigma and other non-muscarinic receptors. Using 2nd messenger assays in cell lines transfected with muscarinic receptor subtypes, PTAC was a M_2/M_4 partial agonist as well as an antagonist at M_1, M_3 and M_5 receptor subtypes. Atypical antipsychotics induce expression of the neuronal activity marker Fos in nucleus accumbens, but PTAC alone was without effect. However, PTAC inhibited d-amphetamine-induced Fos expression in nucleus accumbens, suggesting blockade of DA activities. PTAC did not appreciably increase striatal acetylcholine tissue levels, but blocked the effects of muscarinic agonists and antagonists, suggesting partial agonist effects at M_2 receptors. These findings suggest that PTAC is a novel muscarinic compound that has low affinity for the DA and serotonin receptors thought to mediate the actions of atypical antipsychotic agents. However, it has novel interactions with DA neurons and activity in animal models predictive of antipsychotic activity, suggesting PTAC may represent a new class of antipsychotic agents.

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CHOLINE ALTERS CARDIAC FUNCTION BY ACTIVATING M_3 MUSCARINIC ACETYLCHOLINE RECEPTORS (mAChRs) IN CARDIAC MYOCYTES

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Choline, a precursor and a metabolite of the neurotransmitter acetylcholine, has recently attracted major attention due to its beneficial effects on various neurological disorders including Alzheimer's diseases. Choline has also been reported to be able to induce a novel K^+ current in dog atrial myocytes via stimulation of mAChRs. To elucidate the mechanisms by which choline induces the K^+ current, we performed patch-clamp experiments in isolated canine and guinea pig atrial cells. Choline (0.1-135 mM) activated a voltage-dependent K^+ channel conducting both outward and inward currents in response to depolarizing and hyperpolarizing pulses, respectively. Application of antagonists toward M_1, M_2 and M_4 failed to alter the currents, ruling out the involvement of $M_1/M_2/M_4$ in the current induction. 4-DAMP (an M_3-selective antagonist) substantially inhibited the currents, with an IC_{50} value of about 2 nM. Lengthening of APD was consistently seen after superfusion with choline in a concentration-dependent fashion (0.1-10 mM) in guinea pig atrial preparation with standard microeelctrode techniques. APD_{50} and APD_{90} were increased by 48±5 and 31±8%, respectively, by choline at 1 mM. The heart rate (sinus) was also significantly decreased by choline (25%). The effects of choline were reversed by 2 nM 4-DAMP. Our data indicate that choline can selectively activate M_3 receptors in cardiac cells and plays an important role in modifying membrane repolarization.

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DISTINCT MUSCARINIC RECEPTOR SUBBTYPES LINKED TO DIFFERENT SOURCES OF CALCIUM IN GUINEA-PIG GALLBLADDER

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It was reported that acetylcholine may use either extra- or intracellular calcium sources to cause gallbladder contraction in cats[1]. M_2 receptors were claimed to use calcium influx whereas M_3 receptors were said to be associated with intracellular calcium release. In guinea-pig gallbladder, we obtained some evidence suggesting that M_4 receptors may be involved in contraction[2] and M_2 and M_4 receptor proteins are expressed[3]. The present study aimed to investigate whether intra- and/or extracellular calcium sources are utilized to mediate gallbladder contraction via distinct muscarinic receptor subtypes in guinea-pig. Carbachol (CCh) caused a concentration-dependent contraction in either a 2.5 mm Ca^{2+}, zero Ca^{2+} or 5 mM Sr^{2+} medium with similar pD_2 values (5.75±0.12, 5.10±0.05 and 5.88±0.12, respectively). The amplitude of the maximal contraction in zero Ca^{2+} and 5 mM Sr^{2+} media were 45.8±8.0 and 33.2±6.6 % of that in normal Ca^{2+} medium. Methoctramine exerted slight inhibition on CCh (10^{-5} M)-induced contractions in the presence of 2.5 mM Ca^{2+} (-log IC_{50}=4.89±0.30) and in 5 mM Sr^{2+} media (-log IC_{50}=4.83±0.30). It was significantly more potent in antagonizing contractions in response to 10^{-5} M CCh in the absence of extracellular calcium (-log IC_{50}=5.99±0.19). Atropine also inhibited CCh-induced contractions, however the IC_{50} values were not significantly different from each other in the absence of extracellular or intracellular calcium (-log IC_{50}=6.85±0.11 and 6.88±0.25, respectively). In conclusion, CCh contracts guinea-pig gallbladder via two distinct muscarinic receptor subtypes linked to extracellular calcium influx and intracellular calcium release.

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MOLECULAR SIGNALING MECHANISMS INVOLVED IN ACH-INDUCED CELL PROLIFERATION IN PORCINE TRACHEAL SMOOTH MUSCLE CELLS.

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Excess smooth muscle mass coexist with airway hyperresponsiveness in asthma. Since excess muscle mass is in part related to smooth muscle cell proliferation there is a need for better understanding of mitogen-activated signaling mechanisms. Acetylcholine (Ach) and other muscarinic agonists increase DNA synthesis in Chinese hamster ovary cells, human 1321N1 astrocytoma cells and human cancer prostate cells. In the present study, Ach (10^{-8} - 10^{-4} M) caused a dose- and time-dependent increase in cultured porcine tracheal smooth muscle cell (TSM) proliferation as measured by [^{3}H]thymidine incorporation. The mitogenic effect was mimicked by the stable muscarinic agonist bethanechol (10^{-4} M) and was blocked by atropine (10^{-6} M). Phorbol ester, phorbol-12-myristate-13-acetate (PMA) (10^{-7} M) also induced cell proliferation in TSM. Both Ach- and PMA-induced proliferation were reduced (83% and 96% respectively) by the protein kinase C (PKC) inhibitor calphostin C (10^{-6} M). Approximately 80% of the muscarinic receptors in airway smooth muscle are of the M_2 subtype. We therefore treated TSM with pertussis toxin (160 ng/ml) for 24 hours immediately prior to addition of Ach. Pertussis toxin pretreatment reduced Ach-induced [^{3}H]thymidine incorporation to control. These results suggest that Ach-induced cell proliferation in TSM is PKC-dependent and is also dependent on pertussis toxin-sensitive G_i proteins. (Supported by American Lung Association of Mississippi, HL55544 and NIH grant AA07157-10).

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PERTUSSIS TOXIN ENHANCES THE RELAXANT EFFECTS OF ISOPROTERENOL AND FORSKOLIN IN TRANSNEURALLY STIMULATED GUINEA PIG ILEUM

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Our laboratory has shown that muscarinic agonists can produce a pertussis toxin-sensitive, M_2-mediated contraction by antagonizing the relaxant effects of cAMP generating agents on histamine-induced contraction. In this study, we investigated whether endogenous acetylcholine can mediate contraction through M_2 receptors in field-stimulated guinea pig ileum. Transneural stimulation (100 v, 8 ms duration, 1 pulse every 5 s) produced contractions of 3 to 4 grams that were 4-DAMP mustard-sensitive and pertussis toxin-insensitive. The cumulative addition of isoproterenol and forskolin caused a maximal 52% and 34% reduction, respectively, in the contractile response. Following pertussis toxin-treatment, the maximal inhibitory effect of isoproterenol increased to 83%, and the EC_{50} value for relaxation was 2.2-fold more potent. Similiarly, the the maximal effect of forskolin increased to 79% after pertussis toxin-treatment, and the relaxant potency of forskolin was essentially unchanged. In contrast, pertussis toxin had no effect on the ability of isoproterenol to inhibit contractions elicited by histamine and the H_1 agonist 2-(2-methylaminoethyl)-pyridine. Our results suggest that the increased effectiveness of isoproterenol in pertussis toxin-treated, field-stimulated ileum is due an uncoupling of the M_2 receptor contractile mechanism and not to an enhancement in the relaxant mechanism of isoproterenol. Thus, our data show that endogenous acetylcholine can act on M_2 receptors to antagonize the relaxant effects of isoproterenol and forskolin on the M_3-mediated contractile response.

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FUNCTIONAL EVALUATION OF MULTIPLE EFFECTOR PATHWAYS IN RAT CORONARY VASOCONSTRICTION MEDIATED BY MUSCARINIC RECEPTORS

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In rat coronary resistance arteries, acetylcholine produces a constrictor effect by activating muscarinic receptors. The aim of this study was to elucidate signaling mechanisms for this vasoconstriction. Potassium-arrested isolated rat hearts were perfused with modified Krebs-Ringer bicarbonate buffer at a constant flow rate, and perfusion pressure was monitored as an indicator of coronary vascular resistance. Responses to the muscarinic receptor agonist bethanechol were examined in the absence and presence of specific inhibitors. Bethanechol was given by either bolus injection (100 μl) or infusion (100 μl/min). Vasoconstriction evoked by bethanechol was eliminated by perfusion with Ca^{2+}-free buffer. The voltage-operated calcium channel (VOCC) blocker nifedipine (10 μM) decreased the maximal constrictor response to bethanechol by 61%, while the receptor-operated calcium channel (ROCC) blocker SK&F96365 (10 μM) converted this effect into vasodilation. A combination of SK&F96365 and nifedipine abolished coronary responses to bethanechol. Infusion of bethanechol caused a tonic vasoconstriction without producing tachyphylaxis. This response could be rapidly and reversibly turned off by simultaneous infusion of SK&F96365. These data indicate that VOCCs might be required for vasodilation and ROCCs might be essential to vasoconstriction mediated by bethanechol. The phospholipase A_2 (PLA_2) inhibitor p-BPB (5 μM) suppressed the maximum pressor response to bethanechol by 43%, while inhibition of cyclooxygenase (10 μM indomethacin) or 5-lipoxygenase (10 μM esculetin) decreased this effect by 32% and 34%, respectively. The combined use of these blockers did not cause further inhibition. The protein kinase C (PKC) inhibitor chelerythrine (5 μM) reduced the peak vasoconstrictor response to bethanechol by 79%. Infusion of chelerythrine gradually decreased the tonic vasoconstriction elicited by infusion of bethanechol. We conclude that PKC, ROCCs and VOCCs have major roles in the vasoconstrictor response to bethanechol. Mediators in the PLA_2 signal transduction pathway (such as thromboxane and leukotrienes) have also been implicated but appear to be less important. Supported by NIH HL54633.

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MUSCARINIC RECEPTORS IN RAT UTERUS: EFFECT OF OVARIECTOMY

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In many smooth muscle tissues, mixed populations of muscarinic receptors are present. In rat uterus, M_2 muscarinic receptors have been identified and the presence of another subtype has been suggested (Munns & Pennefather, 1998). As smooth muscle contraction is also known to be modified by the hormonal status (Fernandez et al., 1995), we have characterized the muscarinic receptor(s) present in the rat uterus in the present study and investigated the effect of ovariectomy on contractile responses in this tissue using standard organ bath experiments.

Concentration-contraction curves to carbachol were established in the rat uterus in the absence and presence of several antagonists. Antagonist affinity estimates (pK_B) are summarized in table 1.

Table 1 pK_B values for muscarinic antagonists in sham operated and ovariectomized rat uterus

Antagonist	pK_B (Sham operated)	pK_B (Ovariectomized)
Pirenzepine	6.48±0.25	7.21±0.29
Methoctramine	6.79±0.11	7.49±0.18
Zamifenacin	9.19±0.16	9.18±0.24
AFDX 116	6.26±0.12	6.61±0.21
Tripitramine	7.23±0.12	7.54±0.10
p-F-HHSiD	8.50±0.08	9.06±0.13
Himbacine	7.21±0.18	7.41±0.31
MTx3	< 7.00	< 7.00
PD 102807	< 7.00	< 7.00

Values shown are means ± s.e.m., n≥3.

These results suggest that contractions to carbachol in rat uterus from sham and ovariectomized animals are mediated by a muscarinic receptor whose antagonism profile closely resembles the pharmacologicaly defined M_3 receptor.

Fernandez, A. I., Garcia de Boto, M. J., Gutierrez, M., Cantabrana, B. and Hidalgo, A. (1995). Gen. Pharmacol., 26: 1281-1287.

Munns, M. and Pennefather , J. N. (1998). Br. J. Pharmacol., 123: 1639-1644.

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FUNCTIONAL AND MOLECULAR IDENTIFICATION AND CHARACTERIZATION OF CARDIAC M_3 AND M_4 MUSCARINIC ACETYLCHOLINE RECEPTORS

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Objective: Multiple subtypes (M_1-M_4) of mAChRs have been identified in a variety of primary tissues, but only M_2 subtype has been functionally defined in the heart. We explored mAChR subtypes in cardiac cells. **Methods:** Patch-clamp techniques in isolated single cells, receptor binding assay with membrane homogenates, and RT-PCR methods and molecular cloning techniques with RNA samples from canine atria. **Results:** (1) TMA (tetramethylammonium, 1 μM-0.5 mM) and 4AP (4-aminopyridine, 0.1 mM-1 mM) induced two novel K^+ currents (I_{KTMA} and I_{K4AP}, respectively) conducting at both outward (depolarization) and inward (hyperpolarization) directions, and ACh induced an inward rectifier K^+ current known to be I_{KACh}. These currents were all abolished by atropine. I_{K4AP} but not I_{KTMA} was minimized by treatment with PTX. I_{KTMA} was markedly suppressed by 4-DAMP (an M_3-selective antagonist) but not altered by antagonists for other subtypes, such as pirenzepine (PZ, for M_1), methoctramine (Meth, for M_2) or tropicamide (Trop, for M_4). I_{K4AP} and I_{KACh} were blocked only by Trop and by Meth, respectively. (2) The displacement binding of [^{3}H]-NMS with PZ, Meth, 4-DAMP and Trop yielded K_i values indicating the presence of $M_2/M_3/M_4$ subtypes in canine atrium. (3) RT-PCR confirmed the expression of the $M_2/M_3/M_4$ transcripts in canine atrium. **Conclusions:** (1) Multiple subtypes ($M_2/M_3/M_4$) of mAChRs co-exist in canine atrial cells; (2) Different subtypes of mAChRs are coupled to different K^+ channels.

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ANALYSIS OF MUSCARINIC ACETYLCHOLINE RECEPTOR SUBTYPE EXPRESSION IN ISOLATED SMOOTH MUSCLE CELLS USING RT-PCR

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Recently we have examined the involvement of the different muscarinic acetylcholine receptor subtypes in rat bladder contraction. Through the use of subtype selective antagonists in contractility studies which incorporated both electric field stimulation and carbachol dose response, we have concluded that the post-synaptic M_3 receptors are the predominant mediators of bladder smooth muscle contraction. Through the use of reverse transcriptase polymerase chain reaction (RT-PCR) we were able the detect the presence of m1, m2, m3, and m4 receptor subtype mRNA. Immunoprecipitation of the bladder tissue following the contraction studies revealed 80-90% of total receptor content was m2 as compared to the m3 receptor subtype which was approximately 10%. To further elucidate the location of muscarinic acetylcholine receptor subtypes in smooth muscle cell contraction, we have developed a protocol for performing RT-PCR on single cells isolated from the body and dome of the rat bladder. This highly sensitive technique has revealed that 64% (n=44) of the isolated smooth muscle cells do not express mRNA for muscarinic acetylcholine receptors. Of the remaining 16 cells which express muscarinic acetylcholine receptors, 10 cells (62.5%) express m1, 2 cells (12.5%) express m2, 5 cells (31.3%) express m3, and 1 cell (6.25%) was found to express the m4 subtype. The mRNA encoding the m1 receptor was shown to be coexpressed with the mRNA encoding the m3 receptor subtype in two of the 44 cells examined (4.5%). Characterization of the possible receptor composition expressed by the different smooth muscle cells in the rat bladder will provide further insight into the functional roles of the different muscarinic acetylcholine receptor subtypes.

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LOCALIZATION OF m4 MUSCARINIC RECEPTORS ON DIRECT>>INDIRECT STRIATAL PROJECTION NEURONS.

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The striatum contains two sets of glutamate-activated, GABAergic projection neurons that exert opposing effects on movement. Both sets are modulated by dopamine, which increases movement, and by acetylcholine, which decreases movement. The concentration of m4 receptors in the rat striatum (64 pmol/g) is five-fold higher than in the cortex and hippocampus (Purkerson and Potter, J. Pharmacol. 284, 707, 1998) and the mRNA for m4 receptors is preferentially distributed on "direct" substance P-containing neurons that project to the substantia nigra reticulata (Weiner *et al*, Proc. Nat. Acad. Sci. 87, 7050, 1990; Bernard *et al*, J. Neurosci. 12, 3591, 1992). Direct neurons were identified in Wistar rats after the retrograde transport of fluorescent microbeads injected into the substantia nigra, and were shown not to overlap (<4%) "indirect" striatal neurons identified with microbeads injected into the globus pallidus. Biotinylated m4-toxin was prepared and found to block m4 receptors with >1000-fold specificity (Santiago and Potter, Neurosci. Abstr. 23, 2021, 1997). Projection neurons with m4 receptors were labeled with biotinylated m4-toxin and visualized with avidin-fluorescein. The toxin labeled 85% of the direct neurons and 15% of the indirect neurons. Thus direct projection neurons are modulated by uniquely intense m4 muscarinic neurotransmission. The selective activation of striatal m4 receptors should inhibit the direct pathway, which is overactive in hyperkinetic disorders and underactive in hypokinetic disorders. These studies provide a strong rationale for evaluating the use of an m4 agonist for the treatment of tardive dyskinesia, hemiballismus and Huntington's disease, and for trying an m4 antagonist to treat Parkinson's disease. Supported by AG 06170.

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LONG-TERM ATROPINE TREATMENT ENHANCES SPINAL CORD M3 AND M4 MUSCARINIC ACETYLCHOLINERGIC RECEPTOR DENSITIES, AND PRODUCES ANTINOCI-CEPTION AFTER WITHDRAWAL.

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We have recently demonstrated *(Höglund and Baghdoyan, JPET, 281, 470-477, 1997)* that the spinal cord of the rat contains M2, M3, and M4, but not M1 muscarinic acetylcholinergic receptors (mAChRs). To obtain information on which of these mAChRs that contribute to the modification of nociceptive information at the spinal cord level the present experiment was performed. Male Sprague-Dawley rats (n=10) were treated for 14 days with atropine administered by osmotic minipumps (0.5 mg/kg/h). An equal number of animals was treated with saline given by the same route. During the treatment period the hot-plate and tail-flick latencies were recorded. After 14 days five animals from each group were decapitated and the spinal cords were flushed out from the spines and frozen rapidly on dry ice. The remaining animals were subjected to hot-plate and tail-flick tests the following two days after removal of the minipumps. After withdrawal of the atropine treatment both the tail-flick and the hot-plate latencies increased significantly. Receptor density analysis showed a significant increase of M3 and M4, but not M2 mAChRs. These results implicate a role for M3 and/or M4 mAChRs in spinally mediated antinociception.

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MUSCARINIC RECEPTORS IN THE SUPERFICIAL DORSAL HORN OF THE HUMAN, RAT, AND MOUSE SPINAL CORD.

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Activation of muscarinic receptors in the rat spinal cord produces antinociceptive activity that is blocked by atropine but not by naloxone (Yaksh *et al*, Eur. J. Pharmacol. 117, 81, 1985; Iwamoto and Marion, J. Pharmacol. 269, 329, 1993), and intrathecal neostigmine is effective for postoperative pain (Hood *et al*, Anesthesia 82, 331, 1995). Choline acetyltransferase has been found in nerve terminals abutting primary afferent nerve terminals (Ribeiro-da-Silva and Cuello, J. Comp. Neurol. 295, 370, 1990). ^{3}H-pirenzepine is sharply localized in lamina II of the human cord (Villiger and Faull, Brain Research 345, 196, 1985) and ^{3}H-QNB binding in the rat cord was markedly reduced by dorsal and ventral rhizotomy (Gillberg and Wiksten, Acta Physiol. Scand. 126, 575,1986). We are studying the subtypes and localization of muscarinic receptors that may modulate nociception. Binding assays and autoradiographic studies with human, rat and mouse spinal cord have shown a few m1, moderate m4 and predominant m2 receptors. Binding sites for one nM ^{3}H-NMS were reduced 6% by m1-toxin in the human dorsal horn, 15-30% by m4-toxin in the rat and human dorsal horn, and 60% by gallamine in the rat dorsal horn. Electron microscopy showed monoclonal antibodies for m2 receptors at synapses on dendrites in the rat dorsal horn, indicating that m2 receptors modulate the reception rather than the release of neurotransmitters. Right dorsal L2-S1 rhizotomy reduced ^{3}H-NMS binding in the rat dorsal horn by 10% after one but not seven days, suggesting some muscarinic receptors on primary afferent terminals. Further studies with antibodies and ^{3}H-m4-toxin are necessary to establish whether m4 receptors are localized where they can control sensory transmitters. Supported by AG 06170.

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MUSCARINIC AND β–ADRENERGIC RECEPTORS IN RAT HEART DURING HYDROCORTISONE TREATMENT

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Glucocorticoids affect the expression of β-adrenoceptors (βAR) in certain tissues and cell lines and have differential effects on the expression of β_1 and β_2AR subtypes. Data regarding their effects on cardiac βAR are controversial and the density of muscarinic receptors (MR) in the heart has been reported unaffected by hydrocortisone (HC) treatment. We investigated the effects of the administration of HC to rats (50mg/kg per day), on the densities of MR (determined according to the binding of ^{3}H-QNB) and βAR (determined according to the binding of ^{3}H-CGP 12177) in their hearts. The β_1/β_2 ratio was measured according to competition of radiolabeled ligand with subtype specific antagonists. The activity of adenylyl cyclase (AC) was measured by HPLC determination of cAMP. In the atria, the total density of βAR was elevated after 3-9 days of HC treatment, reaching a peak (240% of control) after 6 days. The elevation concerned both βAR subtypes. The density of MR in the atria was also augmented by HC treatment, reaching a peak (190%) after 6 days. In the ventricles, the total density of βAR was augmented only after 6 days (142%). The density of MR in the ventricles was augmented by 59% after 3 days, and by 23% after 6 days, and returned to the control level after 9 days of treatment. Determinations of the β_1 and β_2 subtypes revealed that the expression of β_1 subtype was suppressed and that of β_2 subtype was enhanced in HC-treated animals. The β_1/β_2 ratio was shifted from approx. 80:20 to approx. 50:50 in the control and HC-treated rats, respectively. The activity of forskolin-stimulated adenylyl cyclase was measured in the atria and found to be little affected by HC treatment (21% increase after 9 days) The finding that glucocorticoid hormone has a profound effect on the density of MR in the heart atria is new and points to the existence of a previously unsuspected way in which the neural control of the heart can be modulated. *Supported by grants from GAUK and GAČR.*

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CARDIOPULMONARY EFFECTS OF THE CHOLINERGIC AGONISTS SB 202026, RS-86, WAL 2014, CARBAMYLCHOLINE AND OXOTREMORINE IN RATS

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Loss of cholinergic neurons in certain brain regions is a neuropathological feature of Alzheimer's disease (AD). However, clinical studies with non-selective direct-acting cholinomimetics have proven to be disappointing due to poor efficacy and/or peripheral cholinergic side effects. Recent evidence suggests that post-junctional M_1 muscarinic receptors are not substantially altered in AD. Thus, agonists for this receptor subtype may be useful to treat the cognitive dysfunction of AD and minimize the undesirable side effects of non-selective muscarinic agents. This study compared the effect of i.v. carbamylcholine (Carb), oxotremorine (Oxo), RS-86 (RS), SB 202026 (SB) and WAL 2014 (WAL) on heart rate and lung inflation pressure in urethane-anesthetized and pithed male Sprague-Dawley rats and determined affinity constants to the human muscarinic receptor subtypes. In anesthetized rats, Carb, Oxo, RS, SB and WAL dose-dependently reduced heart rate. The maximal bradycardic effect of SB and WAL was significantly lower than that of Carb, Oxo and RS.

	Heart Rate - Anesthetized Rats		Heart Rate - Pithed Rats		Ki (μM)		
Agonist	ED_{50} - mg/kg, i.v.	% Decrease[a]	ED_{50} - mg/kg, i.v.	% Decrease[a]	M_1	M_2	M_3
Carb	0.005	71.1 ± 3.2	0.004	75.4 ± 3.2	921.0	2.0	46.0
Oxo	0.007	63.9 ± 3.4	>0.03	12.5 ± 2.2$^+$	8.0	0.44	3.0
RS	0.3	69.7 ± 3.5	0.3	49.1 ± 8.8	45.0	15.0	8.0
SB	0.015	44.6 ± 3.3*	>0.1	20.1 ± 4.3$^+$	0.36	0.08	0.12
WAL	0.9	48.3 ± 3.8*	>3.0	13.3 ± 7.3$^+$	3.0	0.7	0.65

[a] % Decrease = [baseline - agonist dose (mg/kg)]/baseline [Carb (0.01), Oxo (0.01), RS (1.0), SB (0.1) and WAL (3.0)]; * $P<0.05$ versus Carb, Oxo and RS; $^+$ $P<0.05$ Pithed versus anesthetized rats

In pithed rats, bradycardia to Carb and RS was not different from that observed in anesthetized animals, indicating that the bradycardia was due to their peripheral actions. However, the bradycardic effect of Oxo, SB and WAL was diminished in pithed rats, indicating that part of their heart rate lowering effect was due to centrally-mediated actions. Pretreatment of anesthetized rats with hexamethonium (10.0 mg/kg, i.v.) did not shift the Carb dose-response curve, but did produce a 2.3-fold rightward shift of the Oxo dose-response curve, further supporting a central effect of Oxo. Lung inflation pressure of pithed rats was markedly enhanced by Carb (76.3 ± 8.4%, $P<0.05$), Oxo (37.3 ± 11.0%, $P<0.05$), RS (37.4 ± 4.5% $P<0.05$), but not by SB (24.0 ± 4.0%) and WAL (12.8 ± 1.2%). SB had the highest affinity whereas Carb and RS had the lowest affinities for all muscarinic receptors. We conclude that (1) all agonists tested produced a bradycardia in anesthetized rats, although the potency and magnitude was less with SB and WAL; (2) bradycardia to Oxo, SB and WAL was largely due to centrally-mediated actions in contrast to the direct peripheral effect of Carb and RS; (3) Carb, Oxo and RS increased lung inflation pressure producing bronchoconstriction, an adverse effect unlikely to be seen with SB and WAL; and (4) muscarinic receptor affinities did not predict in vivo cardiopulmonary actions of these agonists.

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REVERSAL OF PERTUSSIS TOXIN-INDUCED THERMAL ALLODYNIA BY MUSCARINIC CHOLINERGIC AGONISTS IN MICE

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The intrathecal administration of pertussis toxin produces a long-lasting thermal allodynia in mice. The purpose of the present studies was to compare the antinociceptive effects in normal mice and the antiallodynic effects in pertussis toxin-treated mice of muscarinic cholinergic agonists and cholinesterase inhibitors. In normal mice, the nonselective full muscarinic agonists oxotremorine, H-TZTP and Methylthio[2.2.1], the mixed agonist-antagonist Butylthio[2.2.2], the partial agonists RS86 and pilocarpine, and the cholinesterase inhibitors physostigmine and tacrine produced dose-related antinociceptive effects when tested using a 55 °C water tail-flick test. In mice treated 7 days previously with pertussis toxin (0.3 μg), which induced a condition of thermal allodynia when tested using a 45 °C water tail-flick test, the nonselective full muscarinic agonists oxotremorine, H-TZTP and Methylthio[2.2.1] produced dose-related reversals of the pertussis toxin-induced allodynia whereas the mixed agonist-antagonist Butylthio[2.2.2] produced a partial reversal and the partial agonists RS86 and pilocarpine as well as the cholinesterase inhibitors physostigmine and tacrine failed to reverse the pertussis toxin-induced thermal allodynia. The present results provide further evidence that decrements in pertussis toxin-sensitive Gi/o-protein functioning may be involved in initiating and/or maintaining some neuropathic pain states. Moreover, the present results suggest that muscarinic receptor agonists such as Butylthio[2.2.2] may be useful in the treatment of neuropathic or central pain states which may be due at least in part to inhibitory second messenger system dysfunction.

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BEHAVIORAL AND ELECTROPHYSIOLOGICAL EFFECTS OF THE ANTIPSYCHOTIC-LIKE MUSCARINIC AGONIST PTAC

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The muscarinic agonist xanomeline decreased psychotic-like behaviors in Alzheimer's patients, including delusions and hallucinations, suggesting muscarinic agonists may have antipsychotic activity. We report here that (5R,6R) 6-(3-propylthio-1,2,5-thiadiazol-4-yl)-1-azabicyclo[3.2.1] octane (PTAC) produced robust antipsychotic-like behavioral and electrophysiologic effects thought to be predictive of antipsychotic-like activity. In a conditioned avoidance response task, PTAC and clozapine produced dose-related decreases in avoidance responding, an effect blocked by scopolamine, without producing response failures. Haloperidol decreased avoidance responding but also increased response failures. Moreover, PTAC and clozapine did not produce catalepsy at any dose in contrast to haloperidol which produced catalepsy at doses only slightly greater than those which affected conditioned avoidance responding. Apomorphine-induced climbing was also blocked in a dose-related manner by PTAC in a manner similar to that of clozapine and haloperidol. In rats with unilateral 6-OH-dopamine lesions of the striatum, PTAC blocked dopamine agonist-induced rotations in a dose-dependent manner. In addition, PTAC blocked the increases in locomotor activity produced by d-amphetamine and the schizophrenomimetic phencyclidine. Electrophysiologically, PTAC produced dose-related inhibition of A10 dopamine neurons but did not effect A9 dopamine neurons after acute i.v. administration. Moreover, PTAC continued to produce inhibition of A10 but not A9 dopamine neurons even after 3 weeks of administration. The atypical antipsychotic clozapine similarly inhibits A10 but not A9 dopamine cells, but only after 3 weeks of administration. Unlike other antipsychotic agents, PTAC lacks affinity for dopamine receptors (Bymaster et al., this meeting). Taken together, the present data demonstrate that the muscarinic agonist PTAC produces behavioral and electrophysiological effects similar to those produced by the atypical antipsychotic clozapine and suggest that PTAC may represent a new class of antipsychotic agents.

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MUSCARINIC MODULATION OF HIPPOCAMPAL FUNCTION IN m1 KNOCKOUT MICE

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Acetylcholine (ACh) is a major modulator of hippocampal function. The activation of muscarinic acetylcholine receptors (mAChRs) increases excitability of pyramidal cells by modulating several K^+ conductances. Given the poor selectivity of the pharmacological agents used to characterize these mAChR responses, the molecular receptor subtypes that mediate these actions have not yet been elucidated.

Recently, two novel tools have been developed with which to investigate the role of the molecular m1 mAChR subtype in these functions in the hippocampus. Genetically engineered mice that lack the m1 gene have been shown to lack any detectable level of the m1 receptor protein, and a toxin (m1-toxin) from the green mamba snake has been shown to irreversibly bind and antagonize the m1 receptor. Using electrophysiological recording in hippocampal slices from the m1 knockout mice, as well as hippocampal slices (from wildtype mice) incubated in m1-toxin, we have begun to investigate the impact of the absence of functional m1 receptor on mAChR actions.

mAChRs mediate three major excitatory effects on hippocampal pyramidal cells. These include inhibition of the after-hyperpolarization current (I_{ahp}), a calcium-dependent potassium conductance, inhibition of the M-current (I_m), a non-inactivating voltage-dependent potassium conductance, and induction of an inward current at resting membrane potentials that is likely mediated by the inhibition of a leak potassium conductance.

Patch clamp recording from CA1 pyramidal cells in m1 knockout (KO) mice revealed no significant difference in the degree of inhibition of I_{ahp}, I_m, or induction of the inward current by the mAChR agonist, carbachol, as compared to wildtype (WT) controls. In addition, the m1-toxin was not able to block CCh's inhibition of the I_{ahp}, I_m, or induction of the inward current. These data suggest that the m1 receptor is not involved in increasing CA1 pyramidal cell excitability by mediating acetylcholine's effects on these potassium conductances.

Our future work will focus on investigating the possible roles of m3 and m4 in the cholinergic modulation of these potassium currents.

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GENE-TARGETTED MICE LACKING M5 RECEPTORS: GENOTYPES AND BEHAVIOR.

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The m5 receptor is the least understood of the muscarinic subtypes, and the least amenable to pharmacological analysis, due to the lack of m5 selective ligands. The m5 receptor gene from a mouse cDNA library was isolated, and a targetting vector with a null mutation in the gene was inserted into R1 embryonic stem cells (Nagy et al. 1993). Cell lines showing homologous recombination were used to make chimeric mice. Heterozygous (129 X CD1 strain) offspring of chimeric mice from one cell line were crossed to produce homozygous mice for the m5 mutation, confirmed by Southern blots. The homozygous and heterozygous mice were similar in size, health, behavioral appearance and reproductive status to wild-type mice. Brain regions appeared similar when stained with cresyl violet. Preliminary data indicated a reduction in pilocarpine-induced salivation in knockout mice, consistent with the finding of m5 receptors in submaxillary glands (Flynn et al. 1997). This work was supported by MRC, OMHF and Ontario Schizophrenia Society grants to JY.

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ANTISENSE OLIGONUCLEOTIDE FOR THE M5 MUSCARINIC RECEPTOR IN THE VENTRAL TEGMENTUM REDUCES M5 RECEPTOR DENSITY, BUT NOT SCOPOLAMINE-INDUCED LOCOMOTION.

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Antisense oligos for the m5 receptor infused into the ventral tegmentum increased brain-stimulation reward thresholds in rats (Yeomans et al. 1995). Using similar techniques, rats were infused for 6 days with m5 antisense or m5 missense oligos, followed by recovery. Every 3 days, locomotor activity was tested for 60 min following injections of scopolamine (3 mg/kg i.p.). Although scopolamine reliably increased locomotor activity, m5 antisense or missense infusions produced no significant changes in scopolamine-induced locomotion. We propose that scopolamine blocks m5 receptors as well as other muscarinic receptors, reducing the contribution of m5 receptors to scopolamine-induced locomotion. In another set of rats, m5 receptors in the ventral tegmentum were assayed using the method of Flynn et al. (1997). m5 receptors on the side of the oligo infusion were decreased by 25% by antisense (1.83 fmoles/mg vs. 2.43 fmoles/mg) but not by missense oligos (2.37 vs. 2.36 fmoles/mg) vs. contralateral controls. This work was supported by MRC Canada grant to J.Y. and NIH grant AG12738 to D.D.F.

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MUSCARINIC RECEPTORS AND MEMORY CONSOLIDATION

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Muscarinic toxins (MTs) 1 and 2 from *Dendroaspis angusticeps* venom, show different selectivities for muscarinic receptor subtypes and diverse distribution patterns in the rat brain. MT1 has almost same affinity for m1 and m4, while MT2 show 4 fold higher affinity for m1 than for m4 cloned receptors, with very low affinities for the others (Kornisiuk *et al.*, 1995). MT1 and 2 behave as agonists at the m1 receptor. MT3, more selective for m4 (214 fold) than for m1, and with very low affinity for the others, behaved as an antagonist since it abolished ACh inhibition of adenylyl cyclase (AC) in the striatum (Olianas *et al.*, 1996). MT1 and 2 infused into the dorsal hippocampus of rats after training caused memory facilitation as oxotremorine does, corroborating the role for m1 receptors in memory. Higher doses of MT1 had no effect. This could be due to an interference with m4 receptors. MT3, in two different doses into the dorsal hippocampus of rats after training in an inhibitory avoidance task, resulted amnesic. It was shown that either reduction of cAMP levels or inhibition of PKA after training caused amnesia (Quevedo *et al.*, 1997). If ACh was released, antagonists would allow an increase in cAMP levels and PKA activation, leading to improvement of performance. However, it was reported a different effect of ACh in the olfactory bulb where it increased AC activity. Further research is carried out to clarify the action of m4 receptors on AC activity in different areas of the brain and hence, MT3 action on it. Our results suggest that m4 receptors of the hippocampus are involved in memory consolidation.

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FUNCTIONAL PHARMACOLOGICAL CHARACTERIZATION OF THE MUSCARINIC CHOLINOCEPTOR-MEDIATED INHIBITION OF ADENYLYL CYCLASE IN PRIMARY CULTURED HUMAN BLADDER DETRUSOR SMOOTH MUSCLE CELLS.

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The muscarinic cholinoceptor (mAChR) family is currently comprised of four pharmacologically defined receptors, M_1-M_4. Molecular studies have identified a fifth receptor, M_5, which currently lacks an endogenous pharmacological correlate. mAChRs are known to play a prominent role in bladder contraction (voiding) and mAChR antagonists, such as oxybutynin, have been used to treat bladder hyperactivity ("urge incontinence"). Radioligand binding and immunoprecipitation studies have demonstrated the existence of both M_2 and M_3 mAChRs at a ratio of 3:1 (M_2:M_3) in human bladder detrusor (Yamaguchi *et al.*, 1996, J. Urol. **156,** 1208). Functional tissue bath contraction studies using bladder strips have consistently demonstrated the importance of M_3 mAChRs acting via activation of phospholipase C (PLC). More recently, the role of the M_2 mAChR in reversing β-adrenoceptor mediated relaxation (recontraction), thought to be mediated through inhibition of adenylyl cyclase, has been described in the rat bladder (Hegde *et al.*, 1997, Br. J. Pharmacol., **120**, 1409). The M_3 mAChR-mediated response has also been demonstrated in primary cultured human bladder detrusor smooth muscle cells with robust increases in inositol phosphates accumulation in response to carbachol and antagonist affinities consistent with contraction studies (Harris *et al.*, 1995, J. Urol., **154**, 1241). The current study characterized the muscarinic receptor mediating the carbachol-induced inhibition (pEC_{50} = 6.3) of forskolin-stimulated cyclic AMP accumulation in primary cultured smooth muscle cells prepared from 3 patients. Affinity estimates (pK_b) were obtained from inhibition curves using the Cheng-Prusoff equation and the following antagonists: pirenzipine (<6), darifenacin (<6.8), AQ-RA 741 (8.73), PD 102807 (6.12), MT3 (<5), himbacine (8.17) and tripitramine (8.95). These values are consistent with reported binding values for the human cloned M_2 mAChR as well as functional values obtained from the guinea pig paced atria tissue bath assay (M_2; Eglen *et al*, 1988, J. Pharm. Exp. Ther., **247**, 911) and demonstrate the existence of functionally coupled muscarinic receptors in cultured bladder smooth muscle cells possessing M_2 mAChR pharmacology.

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PHARMACOLOGICAL AND MOLECULAR CHARACTERIZATION OF MUSCARINIC ACETYLCHOLINE RECEPTOR SUBTYPES IN HUMAN HEART

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We have recently identified two new subtypes (M_3 and M_4) of muscarinic acetylcholine receptors (mAChRs) in canine atrium and characterized their physiological functions. It remains undetermined however, whether these subtypes are also present in the human hearts. To explore this possibility, we performed receptor-binding assay with the membrane homogenates prepared from human atria and RT-PCR (reverse transcription-polymerase chain reaction) with RNA samples extracted from human atria. Results from the competitive binding of [^{3}H]-NMS by pirenzepine, methoctramine, 4-DAMP and tropicamide were consistent with the presence of M_2/M_3/M_4 subtypes in human atrial tissues. The competition binding curves for all compounds except for pirenzepine were best described by two-site binding model in our study. The expression of the genes for M_2/M_3/M_4 subtypes was confirmed by PCR amplification of the corresponding cDNA fragments. Our data indicate that multiple subtypes of mAChRs (M_2/M_3/M_4) co-exist in human heart and they are all abundantly expressed at the mRNA level.

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DETECTION OF MUSCARINIC RECEPTOR SUBTYPES IN HUMAN HEART USING A REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

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The expression of genes encoding the m1-m5 muscarinic acetylcholine receptor subtypes in human heart was analyzed using RT-PCR. Moreover, the presence of these subtypes at the protein level was analyzed by in vitro receptor autoradiography. Total RNA was analyzed by RT-PCR following extraction from atria and ventricles of donors' hearts as well as from ventricles of patients with dilated cardiomyopathy (DCM). Our study indicates the presence of mRNA for the M_2 and M_3 subtypes in all compartments of the human heart while M_1, M_4 and M_5 mRNAs were not detected. Only the M_2 (in 6 out of 6 patients) and M_3 (in 4 out of 6 patients) subtypes of muscarinic receptor genes were also expressed in the ventricles of patients with DCM. In line with the RT-PCR studies, autoradiographic analysis confirmed the abscence of M_1 and M_4 receptor subtypes. Thus our binding studies indicate the presence of M_2, M_3 and possibly M_5 receptor subtypes. However, since the M_5 receptor is not expressed, as described above, in the heart it could be concluded that the M_2 and M_3 receptor subtype proteins are only present. Abundance of these proteins in the atria was two to three times higher than in the ventricles. Muscarinic receptor proteins in the ventricles of patients with DCM were significantly increased, decreased or unaltered compared with ventricles of donor hearts.
We conclude that M_2 and M_3 muscarinic receptor subtypes are expressed in the human heart.

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EXPRESSION OF MUSCARINIC RECEPTOR SUBTYPES (M1-M5) AND ADENYLATE CYCLASE SUBTYPES II AND IV IN THE HUMAN AQUEOUS HUMOR OUTFLOW PATHWAY

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Purpose: Aqueous humor is produced by the ciliary processes and leaves the primate eye via the trabecular meshwork (TM) and Schlemm's canal. A reduction in fluid conductivity (i.e. outflow facility (C)) of the TM underlies the increased intraocular pressure present in glaucoma. Human ocular anterior segments with attached TM are capable of responding to cholinergic stimulation by carbachol and other nonselective muscarinic agonists with an increase of C at agonist concentrations of 10-6 M and lower. In order to test the hypothesis that these changes in C might be due to a rise in cyclic AMP, we perfused human anterior segments with carbachol and measured cAMP accumulation in parallel with C and found increases in both cAMP and C. Since it is known that muscarinic receptor subtypes m1 and m3 can increase cAMP via stimulation of adenylate cyclase subtypes II (AC-II) or -IV (AC–IV), we hypothesized that m3 and AC-II or AC-IV would be expressed in aqueous outflow tissue of human eyes. **Methods**: Human eyes were dissected and perfused according to previously described methods. mRNA of m1 to m5 were detected by *in situ* hybridization. mRNA of AC-II and AC-IV were detected by both *in situ* hybridization and RT-PCR. **Results:** m2, m3 and m5 were expressed in both circular and longitudinal cells in CM tissue and cultured CM cells. m3 was detected in the wall of Schlemm's canal. mRNA of AC-II was detected in TM, CM (circular only), ciliary processes (CP), pigmented epithelium (PE), corneal endothelium (CE), corneal stroma (CS), and anterior sclera (AS), but not in cultured human TM, AS, CM, or CS cell lines. mRNA of AC-IV was detected in TM, CM (circular only), CP, PE, AS and cultured AS, but not in CE, CS and cultured human TM, CM and CS cell lines. Outflow facility maximally increased 50% in human eyes perfused with 10-7 M carbachol with a 70% increase in cAMP levels. In contrast, outflow facility did not increase in human eyes perfused with 10-3 M carbachol but cAMP levels significantly increased by 730%. **Conclusions**: Adenylate cyclases type II and IV are present in human aqueous outflow tissues. These cyclases may underlie the outflow facility increases mediated by muscarinic agonists. Supported in part by NIH EYO 7321 and The Glaucoma Research Foundation.

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COMPLEX M_2/M_3 RECEPTOR ANTAGONIST PROFILE IN HUMAN COLON BIOASSAY.

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The human colon, like other smooth muscle (s.m.), expresses both M_2 and M_3 receptors as judged by radioligand binding (Gomez *et al.*, 1992, Biochem. Pharmacol., 43, 2413) but its contractile responses are reported to be M_3 receptor mediated (Kerr *et al.*, 1995, Br. J. Pharmacol., 115, 1518). Here, the interaction between selective antagonists and a non-selective agonist, 5-methylfurmethide (5mef), have been studied on macroscopically normal human taenia coli resected for malignancy (3-10 patients per study). The data were compared with those obtained on guinea-pig gastric s.m. and left atrium assays. The gastric and atrial pK_B values were consistent with expectations for the M_3 and M_2 receptors, respectively. In contrast, the colon data appeared complex, that is, not consistent with the presence of a homogeneous population of either receptor. The pA_2 values (calculated from the first significant shift) for *p*-F-HHSiD and methoctramine were closest to the values obtained in gastric s.m., suggesting M_3-receptor activation. However, in each case, the Schild slope value (b) was significantly less than unity.

Antagonist	Tissue (d.f.)	$pK_B \pm$ s.e. (pA_2*)	b ± s.e.
Pirenzepine	Colon (20)	6.64±0.13	0.90±0.09
	Gastric s.m. (22)	7.03±0.09	0.97±0.06
	Left atrium (16)	6.71±0.07	0.88±0.09
p-F-HHSiD	Colon (39)	(6.98±0.21)	0.51±0.09
	Gastric s.m. (25)	7.57±0.10	0.95±0.07
	Left atrium (18)	5.98±0.09	0.88±0.12
Darifenacin	Colon (45)	(7.74±0.13)	0.96±0.13
	Gastric s.m. (22)	9.29±0.14	0.97±0.08
	Left atrium (31)	7.20±0.05	1.00±0.04
Methoctramine	Colon (55)	(6.07±0.25)	0.52±0.14
	Gastric s.m. (25)	5.40±0.09	1.09±0.23
	Left atrium (33)	7.35±0.07	1.14±0.09

*pA_2 – Schild criteria not fully met

In the presence of darifenacin, the maximum asymptotes of the 5mef concentration-effect curves were reduced although b was not different from unity and a pA_2 value closer to that seen in the left atrium was obtained which would suggest mainly M_2-receptor activation. In addition, some biphasic 5mef curves were noted both in the absence and presence of the antagonists which, along with the low Schild slopes estimated for *p*-F-HHSiD and methoctramine, may suggest that the contraction was mediated by multiple receptors. This contrasts with the original Kerr *et al.* (1995) data, albeit obtained by following a different protocol. Interestingly, Kerr & Hillier (1997, Br. J. Pharmacol., 120, 196P) subsequently reported an atypical antagonist affinity profile in binding studies on the same tissue.

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FACILITATION OF ACETYLCHOLINE RELEASE FROM MYENTERIC NEURONES BY α,β-METHYLENE ATP CAUSES A CONTRACTION OF THE GUINEA-PIG ILEUM VIA POSTJUNCTIONAL M_3 RECEPTORS

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Previous studies have shown that the guinea-pig ileal longitudinal smooth muscle (GPI) possesses neuronal muscarinic M_1 receptors whose activation leads to an increase in acetylcholine (ACh) resting release (Kilbinger and Nafziger, NSAP 328: 304, 1985). It has also been reported that P2X-like receptors are located on the soma-dendritic region of myenteric neurones in GPI, the stimulation of which causes release of ACh and, subsequently, a tetrodotoxin- and atropine-sensitive contraction (Kennedy and Humphrey, EJP 261: 273, 1994). The aim of the present study was to characterize the muscarinic receptor subtype mediating the indirect, ACh-induced contractions of the GPI to the P2X receptor agonist α,β-methylene ATP (mATP) by means of subtype-prefering muscarinic antagonists.

Preparations of GPI were incubated at 37°C in modified Krebs buffer (1.3 mM Ca^{2+}; supplemented with 70 nM physostigmine). Tetrodotoxin-sensitive (1 μM) contractions to single doses of mATP (3 μM) or electrical field stimulation (EFS; 0.1 Hz) were measured isometrically. To give a numeric value to the potencies of the antagonists, concentrations which produced 50 % inhibition of contraction to mATP or EFS (pIC_{50} values) were determined.

The muscarinic receptor antagonists atropine, p-F-HHSiD, himbacine and pirenzepine concentration-dependently reduced and finally abolished contractions elicited by mATP and EFS, with potency decreasing in that order (see Table). Excellent correlations were found comparing the antagonist potencies from mATP experiments (pIC_{50}-mATP) with the respective pIC_{50} values obtained in EFS experiments and with pA_2 values derived from Schild analysis in GPI, using exogenous muscarinic agonists: r (pIC_{50}-mATP vs. pIC_{50}-EFS) = 0.993; r (pIC_{50}-mATP vs. pA_2) = 0.992. These results demonstrate that neurogenic cholinergic responses elicited by mATP or EFS in GPI are mediated by postjunctional muscarinic M_3 receptors. *Supp. by DFG.*

Muscarinic antagonist	pA_2 M_3	pIC_{50} mATP	pIC_{50} EFS
Atropine	8.99[2)]	7.76	8.44
p-F-HHSiD	7.84[1)]	6.93	7.42
Himbacine	7.10[2)]	6.01	6.69
Pirenzepine	6.88[1)]	5.88	6.39

[1)] Lambrecht et al., EJP 152: 193, 1988.

[2)] Eltze et al., EJP 238: 343, 1993.

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STEREOSELECTIVE INTERACTION OF TRIHEXYPHENIDYL AND RELATED COMPOUNDS WITH MUSCARINIC RECEPTORS

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The enantiomers of the antiparkinsonian drug, trihexyphenidyl (THP), exhibit high stereoselectivity (up to 400-fold) at muscarinic receptors, the (R)-isomer being the eutomer with a selectivity profile of M_1(pK_i: 9.0) = M_4(8.8) > M_3(8.1) > M_2(7.7) (Waelbroeck et al., EJP-MPS 227: 33, 1992). In this study, the enantiomers of compounds related to THP were investigated for their affinities at M_1-M_4 receptors. In order to obtain information on the stereoselective interaction of these compounds with muscarinic receptors, we replaced the phenyl or the cyclohexyl ring by a cyclohexyl or a phenyl ring, respectively (→ achiral diphenyl and dicyclohexyl analogues). The affinities (pA_2 or pK_i values) were determined in functional assays at M_1-like (rabbit vas deferens), M_2 (guinea-pig atria) and M_3 receptors (guinea-pig ileum) as well as in radioligand binding studies at M_4 receptors (rat striatum). All compounds behaved as competitive antagonists. Exchange of the OH moiety in (R)-THP by a CH_2OH or a $CONH_2$ group decreased affinity (up to 200-fold), stereoselectivity and receptor-selectivity [M_1=M_3(=M_4)>M_2]. The achiral diphenyl and dicyclohexyl derivatives exhibited lower affinities and receptor-selectivities than the corresponding eutomers of THP and of the CH_2OH analogue, but higher affinities than the corresponding distomers. The sums of the affinity differences {[pA_2(eutomer)-pA_2(diphenyl)]+[pA_2(eutomer)-pA_2(dicyclohexyl)]} were very similar to the experimentally obtained eudismic indices [pA_2(eutomer)-pA_2(distomer)]. With regard to the concept of the four-binding-site model (Waelbroeck et al., EJP-MPS 189: 135, 1990), these results suggest that the stereoselective interaction of the enantiomers with an OH or CH_2OH group is based on opposite binding to site 1 and site 2 by the phenyl and cyclohexyl ring. In contrast, the diphenyl analogue of THP with a $CONH_2$ instead of the OH moiety (fenpipramide) displayed up to 220-fold higher affinities than the corresponding eutomer (phenyl/cyclohexyl-type), a binding behaviour, which could not be explained by the concept of the four-binding-site model. It is suggested that these amides interact with other subsites of muscarinic receptors than THP and its CH_2OH analogue.

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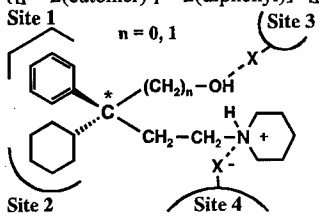

Interaction of (R)-THP (n=0) and analogues with muscarinic receptors

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1. T.M. DAWSON and S.H. SNYDER, J. Neurosci. **14** 5147-5159 (1994).
2. G.R. UHL, *Methods in Neurotransmitter Receptor Analysis,* H.I. Yamamura, S.J. Enna and M.J. Kubar (Eds), 219-244, Raven Press, New York (1990).

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